STUDIES ON HUMORAL IMMUNE RESPONSES IN KALA-AZAR INFECTION AND ANALYSIS OF IMMUNOREACTIVE ANTIGENS AND MOLECULAR CHARACTERIZATION OF LEISHMENIA DONOVANI

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DOCTOR OF PHILOSOPHY

BY

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This thesis is submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) under the Faculty of Post-Graduate Medical Sciences & Research, University of Dhaka. This work has been carried out at the Department of Microbiology NIPSOM & BIRDEM. This is an original and innovative type of work and to the best of my knowledge it has not been done anywhere in Bangladesh.

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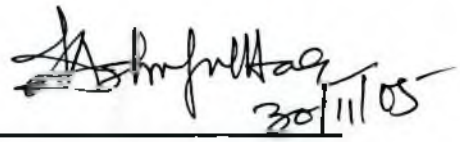
Certification of thesis work

The thesis titled "***Studies on humoral immune responses in Kala-azar infection and analysis of immuno-reactive antigens and molecular characterization of Leishmania donovani***" is submitted by Dr. Md. Zaforullah Chowdhury, Reg. No. 8/2001-2002 in fulfillment of the requirements for the degree of Doctor of Philosophy (Ph. D) under the University of Dhaka. This is an original and innovative type of work and so far has not yet been done elsewhere in the country. This work is interesting to us and is being approved by:

1. 

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DEDICATED TO

MY PARENTS

Their honesty encouraged me to build up my carrier as a

Doctor & Scientist to serve the nation

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Abstract

Kala-azar (KA) or visceral leishmaniasis (VL) is caused by the parasite called *Leishmania donovani*. The disease is endemic in Bangladesh and in the region. It is a chronic infection and has profound effect on immune system of the body. The present study was carried out in a Kala–azar endemic rural area (sub-district or Upazilla) to know the present magnitude and status of leishmania infection and kala–azar, to analyze *L. donovani* specific humoral immune responses and to assess the effectiveness of molecular techniques to diagnose and monitor kala-azar cases. Attempt was also made to detect the parasite in sand fly vectors.

The study was designed in two parts. Sero-epidemiology was conducted on population of KA endemic rural areas and suspected KA patients were recruited for immunological and molecular studies.

Sero-epidemiological study was conducted in Fulbaria Upazilla under Mymensigh, an endemic district of Bangladesh, which is about 200 kilometer northeast of Dhaka. Multistage sampling method was followed for the selection of the study area and population. A total of 1844 people were included from 449 households of 10 villages. A validated ELISA test, using K-39 antigen, was employed to detect anti-leishmania IgG antibodies for determination of leishmania sero-positive cases.

Out of 1844 household members, 114 (6.2%) individuals were sero-positive or exposed to *L. donovani*. Out of these 114, 70 (3.8%) had active kala-azar disease as determined by clinical features and confirmed by further laboratory investigation. Based on the sero-positivity rate, an estimated 26,598 and 12,869 people had exposure to *L. donovani* infection and active kala-azar respectively in entire Fulbaria Upazila of Mymensing district having a population of 428,998. About 18% of the households and almost all age groups were affected. Kala –azar was common among low socio-economic group.

Analysis of antibody responses of cases suffering from active kala azar revealed that total IgG and IgG1 antibody were most pronounced. Other IgG subclasses namely IgG2, 3 and 4 were not significantly raised following infection. The findings indicate that *L. donovani* selectively modulates subset of T helper lymphocytes. Sensitivity and specificity of detecting active kala azar cases by using IgG and IgG1 as markers were 97% and 100% respectively. Majority of kala-azar cases showed consistent gradual decline of total anti-leishmania IgG and IgG1 antibodies over 9-month period following treatment. Out of 78 cases which were followed for 9 months, 94% patients became IgG negative (cut off OD < 0.87) by 9 months while for IgG1 it was 80%.

Detection of L. *donovani* specific nucleic acid from peripheral blood mononuclear cells by polymerase chain reaction (PCR) was attempted. Out of 131 cases, 33 parasite positive cases were selected randomly for PCR and DNA study. Of the 35 buffy coat samples, all were positive by PCR having bands of DNA at 354 bp by the primers constructed from kinetoplast. Buffy coat from 8 kala azar patients after 15 days of treatment were also studied. All of them became negative by PCR indicating its value as a tool to monitor the effectiveness of treatment. Buffy coat specimens were all negative by culture in NNN medium.

PCR of extracted DNA from sand flies (*P. argentipes*) revealed 354 bp bands similar to buffy coat and bone marrow samples. This is the first demonstration of *L. donovani* parasite in sand fly vector in Bangladesh.

Western blot analysis of parasite antigen reveled that majority of the serum-recognized bands around the 60 to 63 KDa and 20-22 KDa regions.

Therefore, the present study demonstrated that the kala-azar is an emerging public health problem in Bangladesh and serology and molecular techniques like PCR are useful tools in diagnosis and monitoring of the treatment success.

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List of Abbreviations

ACP	Acid Phosphatase
Addns ions	Additonal ions
ALAT	Alanine amino transferase
ASAT	Asparatate amino transferase
AT	Aldehyde test
BIRDEM	Bangladesh Institute of Research & Rehabilitation Endocrine Metabolibolic Disorder
° C	Centigrade
CA	Cellulose acetate
CAF	Cellulose acetate electrophoresis
CD	Clusture differentiated
CFT	Complement fixation test
CL	Cutaneous Leishmaniasis
Cm	Centimeter
CEMEM	Complete Eagle's minimum essential media
CMI	Cell mediated immunity
DAT	Direct agglutination test
Dev buffer	Developer buffer
DDT	Dichloro diphenyl trichloroethane
DMCH	Dhaka Medical College Hospital
DMSO	Dymethyl sulphoxide
DTT	Dithiothreitol

EACA	E –amino caprnic acid
E. C.	Enzyme commission
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent assay
EMEM	Eagle's Minimum Essential Media
g	Gravity
gp63	Glycoprotein 63 kilo Dalton
G6PDH	Glucose 6 phosphate dehydragenase
GPI	Glucose phosphate isomerase
HCH	Hexachlorocyclohexane
IEDCR	Institute of Epidemiology Disease Control and Research
IFN	Interferon
IFAT	Immuno fluorescence antibody test
IL	Interleukine
IPGMR	Institute of Post Graduate Medicine and Research
I. U.	International unit
KA	Kala-Azar
Kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
LDH	Lactic dehydrogenase
LPG	Lipophosphoglycan
µm	Micrometer
µgm	Microgram

MCL	Muco-cutaneous Leishmaniasis
MDH	Melate dehydrogenase
mg	Milligram
Mg acetate	Magnesium acetate
$MgCl_2$	Magnesium chloride
mins	Minutes
ml	Milliliter
mM	Milli Molar
MPI	Mannose phosphate isomerase
MTT	Methyl thiozole tetrazolium
MW	Molecular weight
NNN	McNeal, Novy, Nicolle
NAD	Nicotinamide adenine dinuçleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NIPSOM.	National Institute of Preventive and Social Medicine
Na_2EDTA	Disodium ethylene diamine tetraacetic acid
Na_2HPO_4	Disodium monohydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NH	Nuclease hydroxide
PBSS	Proline balanced salt solution
PGM	Phosphoglucomutase
6PGDH	6 Phosphogluconate dehydragenase
pH	Negative logorithm of H^+ concentration

PKDL	Post kala –azar dermal leishmaniasis
PMS	Phenozine methosulphate
Sb_2	Sodium stibogluconate
Tris	Tris hydroxy methyl aminomethane
T_H	T Helper cells
VL	Visceral leishmaniasis
WHO	World Health Organization
(-)	Cathode
ZCL	Zoonotic Cutaneous Leishmaniasis

CHAPTER I

INTRODUCTION

1.1 Introduction:

Leishmaniasis is a group of protozoal diseases caused by parasites genus– *Leishmania*. This parasite is transmitted to humans by the bite of infected sand flies. *Leishmania* is responsible for various syndromes in humans such as kala-azar (KA) or visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), zoonotic cutaneous leishmaniasis (ZCL) and post-kala-azar dermal leishmaniasis. KA or VL is endemic in many parts of Bangladesh and is caused by *L. donovani*. KA is characterized by fever, splenomegaly, hepatomegaly, weight loss and anemia (WHO, 1984).

Besides Bangladesh, visceral leishmaniasis is widely distributed through out the world, viz. India, China, South America, South Africa, and in Mediterranean countries. Nine out of ten cases occur in Bangladesh, Brazil, India and Sudan. Currently, the leishmaniasis is endemic in 88 countries. The large number of endemic countries illustrates the global importance of the problem. The overall prevalence is 13 million cases and the estimated population at risk is about 350 million. A common estimate of the incidence per year is 1.8 million newly reported cases (WHO, 1996).

Resurgence of KA or VL is a serious health problem of the developing countries including Bangladesh. Available records indicate the presence of this disease in endemic form in Bangladesh as early as fifth decade of the nineteenth century (Birley, 1993). Gramccia *et al.*, (1953) reported that about

8.35% children below the age of 15 yrs were positive for KA by aldehyde test between January to July 1950 in endemic districts in Bangladesh.

But during 1960s, the disease almost disappeared due to malaria eradication activities. A resurgence of KA was first noted in early 1970 when Rahman *et al.,* (1983) reported 59 parasitologically confirmed cases of KA out of 218 suspected cases between 1968 to 1970 and 1973-1980 from different parts of endemic areas of Bangladesh. A recent study reported the incidence of KA as 0.9 cases/ 1000 population in the endemic areas of Bangladesh and 31 out 64 districts are already affected (Chowdhury *et al,* 1988; WHO, 1988).

Kala- azar is associated with leishmanial antigen specific suppression in the cell mediated immune components of the host (Carvalho *et al.,* 1989). KA patients, after recovery regain their normal cellular immune functions and usually become immune to a subsequent attack. Preferential activation of IL-4 producing Th-2-like cells may be involved in the exacerbation of human VL, whereas IFN-gamma-producing Th1 cells may protect the host from sever disease. The course of infection appears to be determined by the pattern of the lymphokines produced by leishmania-reactive CD4 T cells. Th1 cells mediate delayed type hypersensitivity (DTH) reaction while Th2 cells cannot. Both Th1 and Th2 cells can provide help for antibody production; however Th2 cells are more efficient. Th1 cells provide help in antigen-specific secondary responses with primed B cell population. However a major difference in help provided by

Th1 and Th2 cells for antibody production is their ability to stimulate different antibody isotypes. Th1 clones induce more IgG2a whereas Th2 clones induce production of IgE and IgG1. The selective induction of antibody isotype appears to be due to cytokines secreted by each cell subset. IL-4 from Th2 cells enhances IgE production; in contrast IFN could switch the isotype from IgE to IgG2a. Therefore IFN and IL-4 can reciprocally regulate B cell immunoglobulin production (Snapper *et al.*, 1987).

Gammaglobulins are produced by the B-lymphocytes and are the major components of humoral immune response to infectious or foreign antigens. Grey and Kunkel (1964) demonstrated that there are four subclasses of immunoglobulin G (IgG) namely IgG1, IgG2, IgG3 & IgG4. The discovery of IgG subclasses has further led to the realization that various gammaglobulin deficiencies may be associated to IgG subclass abnormalities. The production of antibodies in different IgG subclass during infection is dependent on the etiologic agent involved (Del Prete *et al.*, 1991). Filarial-specific IgG4 seems to correlates with active *Wucheria brancrofti* infection (Kwan-Lim 1990) and duration of infection IgG4 has been reported to be a useful marker of *Brugia malayi* infection in Malaysia (Rahman 1979) and Indonesia (Harrbrink *et al.*, 1995). Recent study by Ghose *et al.*, (1995) suggests that certain leishmanial antigens preferentially stimulate the particular IgG subclass(s), depending on the nature of such antigens or their epitopes in Indian KA and PKDL. Nevertheless, no information is available on the effect of conventional

treatment of KA on the state of IgG subclasses in the cured persons. Therefore, identification of the specific IgG subclass(s) might be helpful for early and accurate diagnosis of active stage of KA. The subclass determination of anti-leishmanial antibodies will help in early diagnosis of the disease and distinguish active cases from old cases. However, the determination of the subclass specific antibody response may be useful not only for a diagnosis but it is important for a better understanding of the mechanism of the immune responses involving B and T cell.

Therefore, we are interested to investigate leishmanial antigen-specific IgG subclass antibody response in order to find possible differences in the IgG subclass pattern in the different phases of infection. Further, the kinetics of IgG subclass profile after treatment of established VL patients will be evaluated in the present study.

At present, diagnosis is based on demonstration of parasites in aspirations of spleen (98% positive), bone marrow (54.86%) or lymph nodes (64%) and serological tests (WHO, 1984). Different serological tests commonly used for diagnosis of kala-azar are Complement Fixation test, Immunoflourescence agglutination test, Enzyme linked immunosorbent assay and Direct agglutination test. CFT becomes positive in the early stage of infection (in 3 weeks); it is also a non-specific test and sensitivity of this test varies with the

method of antigen extraction. In addition, due to its complicated procedure the test can only be performed in reference laboratories.

In Bangladesh and other countries, several studies have compared different diagnostic tools for the diagnosis of *L. donovani* infection and found IFAT, ELISA & DAT equally sensitive and specific (Muazzam *et al,* 1990; Alam *et a.,* 1996). ELISA was positive in 100% parasitologically positive KA cases and negative in the control group (Alam MJ, 1996). Similar finding was also reported by Muazzam *et al in (*1990). IFAT was found to be 100% sensitive and 94-100% specific; ELISA was 96.5-100% sensitive and 87.3-98.6% specific (Jahn *et al.*, 1983; Harith *et al.,* 1987; Muazzem *et al.*, 1990).

Complement fixation test was introduced more than 60 years ago for the diagnosis of VL (Smith *et al.,* 1984). Although, CFT becomes positive in the early stage of infection (in 3 weeks) it is a non-specific test. The test has shown to be of limited value in routine laboratory diagnosis (Duxbury *et al.*, 1964) and in large-scale epidemiological studies (Hommel *et al,* 1978). Sensitivity of this test varies with the source and method of extraction of antigen and al (Aikat *et al,* 1979). So with different batches of antigen and false positive result are encountered. Due to its complicated procedure the test can only be performed in reference laboratories (Rahman *et al.*, 1979).

Harith *et al,* (1988) observed that the diagnostic performance of DAT was similar to that of IFAT; both being 100% sensitive and specific. In Bangladesh, Chowdhury *et al,* (1991) found the sensitivity and specificity of DAT to be 96.6% and 97.2 respectively and supported its reliability in diagnosis of VL. El-Masum *et al.,* (1995) showed that DAT was100% positive in parasitologically positive VL patients of Bangladesh.

Current diagnostic methods based on parasite detection (stained smears, culture, and histopathology) and immunological methods have several limitations, including low sensitivity and specificity. Procedures for demonstration of the parasite in spleen or bone marrow in KA and in skin lesions in PKDL are invasive and often not sensitive enough. Immunological methods fail to distinguish between past and present infections and are not reliable in the case of immuno-compromised patients (Badaro *et al,* 1996). Furthermore, neither of these methods addresses the problem of species identification, which is important for determining appropriate treatment regimens and designing control measures. Procedures involving the use of monoclonal antibodies, isoenzyme and zimoderm analysis, and DNA hybridization have to be restored (Grimaldi *et al,* 1987; Rodriguez *et al,* 1994). Most of these procedures are tedious and require massive cultures of parasites. There is, therefore, an urgent need to develop diagnostic procedures that are simple, sensitive, and specific.

Among the new and promising diagnostic tools molecular biology such as polymerase chain reaction plays a great role. Several primers have been developed to amplify specific regions of DNA from *Leishmania* parasites. Recently many investigators have successfully used polymerase chain reaction (PCR) to diagnose VL cases using bone marrow and lymph node aspirates. Leishmania DNA has been detected by PCR in these samples. PCR has provided a diagnosis of KA with 96% sensitivity using patient whole blood samples instead of bone marrow or spleen aspirates that are obtained by invasive procedure (Poonam *et al.,* 2001). PCR detection of parasite from bone marrow was also reported by Shamsuzzamans, *et al.,* (2001) from Bangladesh. The sensitivity of PCR for the detection of *Leishmania donovani* DNA in blood samples reported as 70% to 90% (Adhya *et al.,* 1995; Nuzum *et al.,* 1995). PCR detection of *L. donovani* in any clinical samples can be used as a prognostic marker for the evaluation of successful treatment or relapse cases (Osman *et al.,* 1998).

No systematic research has been done to diagnose *L. donovani* infection from peripheral blood samples using PCR in Bangladesh. Therefore, we are interested to investigate the utility of PCR in the diagnosis of VL from peripheral blood samples without resorting to an invasive method like bone marrow or spleenic aspiration. In this study, we also intend to investigate the effectiveness of PCR method to detect the parasite sp. DNA in peripheral blood to monitor the effect of treatment.

By using western blot apart from PCR method, Mary et al (1992) has demonstrated that immunoblot analysis of antileishmania antibody is a promising technique to diagnose VL due to *L. infantum*. They have shown that antibody to 14-16 KD polypeptide has great specificity.

Ghosh *et al,* (1995) observed that immunoblot analysis with IgG class-specific reagents show variable patterns of reactivity of Indian VL and PKDL sera, although certain common bands around 60- and 63-kD regions were discernible.

It is reported that anti-leishmanial antibodies produced following infection specifically recognize certain leishmanial antigens by *L. donovani* (Ghose *et al.,* 1995). Analysis of crude preparation of antigens revealed those 22-28 kDs and 60-63 kD bands are recognized by the different subclasses of IgG antibodies. However, no study has yet demonstrated the change of recognition of these bands with the progression of the disease.

During the investigation of outbreak of kala-azar in a northeastern district (Tangail) of Bangladesh in 1987, the sand fly fauna was checked. The sand fly species as identified in the localities were *P. argentipes, Sergentomyia babu babu, S. barraudi* and *S. shortii* (Masum *et al.,* 1990a). Another outbreak occurred in northern Thakurgaon district in 1987 and the collected sand flies were *P. argentipes, S. babu babu and S. barraudi* (Masum *et al,* 1990b).

During those collections, two species of *Phlebotamus, P. argentipes* and *P. papatasi* and three species of *Sergentomyia, S. babu babu, S. shorttii and* s. *barraudi* were identified.

Two species of *Phlebotamus, P. argentipes* and *P. papatasi* and five species of *Sergentomyia, S. babu babu, S. baghdadis, S. shorttii, S. barraudi* and a new species were identified from fixed localities in Dhaka and Rajshahi division of Bangladesh. Some studies were tried to detect *L.donovani* parasite in the sand fly by dissection but failed and so far no studies could demonstrate the presence of parasite in these vector in Bangladesh. The detection of *Leishmania donovani* DNA in sand flies caught in Indian KA patients' dwellings was also studied using PCR (Mukherjee *et al.*, 1997). Therefore, we are interested to investigate the utility of PCR in the detection parasite in sand flies caught in KA patient dwellings of the endemic localities by PCR method

Early diagnosis of VL is necessary to reduce the mortality. The conventional direct diagnostic methods of VL are mainly based on demonstration of parasites in biopsies or aspirates from infected spleen, bone marrow and lymph nodes. Their sensitivities are not so high. A number of indirect methods such as ELISA, IFAT and DAT have been developed. Diagnosis of VL by detecting antibodies in sera has some disadvantages as it shows some false positive and false negative result and the antibody in the blood persist for a long time. So it is difficult to differentiate old and new cases and also relapse and re-infected

cases. Recently polymerase chain reaction (PCR) and ELISA have been successfully used and to solve these problems and also to diagnose VL cases from the peripheral blood, bone marrow, and lymph node aspirates.

In order to make suitable plans to control the disease and to understand the epidemiology, identification of the species is important. It is also necessary to develop diagnostic reagents for a particular endemic area. Monoclonal antibodies based on ELISA are the tool used to identify species from the cultured parasites. A few reports on identification of the New World *Leishmania* species directly from the clinical samples of VL patients using PCR have been published. Although a few reports on recent status of kala-azar and its causative agents in Bangladesh have been published but no study or systematic research for diagnosis of Leishmania from blood samples using PCR has been tried. So the present study is designed to assess the value of PCR in the diagnosis of Kala-azar and using it as a tool to differentiate old and new cases and relapse and re-infected cases from blood samples, which is a non-invasive method. This study also attempted to analyze L. donovani specific IgG subclass antibody distribution before and after treatment of patients suffering from KA/VL, to find out the kinetics of IgG subclass profile after treatment of established VL patients, to do the analysis of the leishmanial antigen(s) with respect to their pattern of reactivity to different IgG subclasses by immunoblotting techniques.

As ELISA and PCR were compared with the Gold standard i.e. direct microscopic finding of *Leishmania Donovani* in the Bone marrow, so it is a reliable method. Though PCR is not accessible to the different health facilities but it can be done in a limited scale to see the effectiveness of PCR in detecting *leishmania donovani* from blood samples. PCR will be also tried to investigate the patients who are suffering several times with kala-azar whether they are re-infected or relapse cases.

1.2 Objectives:

It is anticipated that like other infectious diseases there will be antigen specific IgG subclass response in L. Donavon infection, which will be related to different phases of infection. Also, it is assumed that such immune response will be directed to a specific leishmanial antigen. Therefore the objective of our present study is.

1. To analyze L. donovani specific IgG subclass antibody distribution before and after treatment of patients suffering from KA/VL;
2. To find out the kinetics of IgG subclass profile after treatment of established VL patients;
3. To do the analysis of the leishmanial antigen(s) with respect to their pattern of reactivity to different IgG subclasses by immunoblotting techniques;
4. To assess the effectiveness of PCR as a diagnostic and prognostic tool
5. To assess the effectiveness of PCR as a tool for identifying the presence of parasite in the vector from the patient of endemic area.
6. To find out the socio-demographic status of the study population and
7. To determine the sero-prevalence of kala-azar infection in an endemic rural community

CHAPTER II

REVIEW OF LITERATURE

2. Review of Literature:

Leishmaniasis is a group of infection of viscera, skin, and mucous membrane caused by protozoa of the genus *Leishmania* that are transmitted by sand flies of the genera *Phlebotomus* (Old World Leishmaniasis) and *Lutzomyia* (New World leishmaniasis) (Chulay, 1991).

The clinical manifestations of the leishmaniasis depend on complex interactions between the virulence characteristics of the infecting *Leishmania* species and the immune responses of its host. The result is a spectrum of disease ranging from localized skin lesions to diffuse involvement of the reticuloendothelial system. Human disease has traditionally been divided into three major clinical syndromes visceral, cutaneous and mucocuteneous leishmaniasis; however, a number of variants exist. Furthermore, a single *Leishmania* species can produce more than one clinical syndrome and each syndrome is caused by multiple species.

2.1 Visceral Leishmaniasis:

Visceral leishmaniasis (VL) or kala-azar is a disease that is insidious in origin, slow in development and fearful in effects. Visceral leishmaniasis (VL) classically includes fever hepatosplenomegaly, pancytopenia and hypergammaglobulinaemia. Untreated VL is frequently fatal primarily because of bacterial super infections (Manson-Bahr *et al.*, 1991).

2.2. Historical Review:

The organism in VL or kala-azar was first described in 1903 by Sir William Leishman who examined the spleen of a British soldier stationed at Dumdum near Calcutta, India. Later the same year, Charles Donovan verified this finding and the organism are often called Leishman-Donovan (LD) body (Chulay, 1991). The genus Leishmania was created by Ross in 1903 to include *Leishmania donovani.* In India, the disease is known as kala-azar, meaning "black sickness or fever" as the disease turns the color (pigmentation) of the skin black, the word '*kala*' means '*black'* and '*azar'* means '*deadly'*, thereby, signifying a fatal illness (Chatterjee, 1982). In 1904, Roger observed the conversion of amastigotes to promastigotes in culture (Chulay, 1991). A similar parasite were observed in a disease of children in the mediterrenean countries by Nicolle in 1908, proposed the name of infantile kala-azar for this disease and designated the parasite as *L. infantum* (Chatterjee, 1982). Further investigation revealed that it was a strain of *L. donovani.* The parasite of South American VL originally named as *L. chagasi* in 1937, was also found to be indentical to *L. donovani.* Alder and Theodor found Promastigotes in sand flies in 1925 (Chulay, 1991). In 1940, it as was demonstrated the *Plebotomus argentipes* was the vector of India kala-azar (Birley, 1993) and in 1942, in India kala-azar was transmitted experimentally to human volunteer by sandfly bite (Swaminathan *et al.,* 1942).

Irregular epidemic waves have swept through Assam, Bengal and Bihar since the 1800s (Birley, 1993) with a frequency of 15-20 years. In 1890-1900 an epidemic swept Assam, which depopulated whole villages and reduced populations over large areas. In 1917 another epidemic started in Assam and Bengal and reached its height about 1925 and mysteriously subsided, unit by 1931, it was almost gone. In 1937, a new outbreak began in Bihar. Before the Second World War, kala-azar was endemic in Assam, Bengal (a part of which is now Bangladesh), Bihar and some other parts of India subcontinent. Due to insecticide spraying as a part of malaria eradication campaign in 1958-1964, kala-azar almost disappeared from eastern states of India and Bangladesh but with the cessation of insecticide spraying there has been a resurgence of VL in these regions (Thakur *et al.*, 1981; Rahman *et al.*, 1983).

2.3 Geographical Distribution, Prevalence and Incidence:

Visceral leishmaniasis (VL) has a worldwide distribution in more than 88 countries in four continents. Visceral leishmaniasis is endemic in Europe in Mediterranean and neighboring countries; in Asia, north and west China, Middle East, Central Asia, India and Bangladesh; in Africa on the Mediterranean region, East and part of West Africa; and Central and South America (Desjuex, 1993).

Although it is thought to be under reported, rough estimate suggests that 350 million people are at risk of the infection and approximately 12 million are

infected with Leishmania species (Ashford *et al.,* 1992). Annual incidence of VL is around 500,000 cases, ninety percent of all VL cases occur in Bangladesh, India, Nepal and Sudan (Desjuex, 1993). Post kala-azar dermal leishmaniasis (PKDL) is sequelae of VL. It occurs in about 20% cases of VL in India and Bangladesh while only in 2% cases in Africa (Manson–Bahr *et al.,* 1991).

In the post decade, there has been a resurgence of VL in Bangladesh. The current prevalence is estimated to be at least 30,000 cases. The total population considered at risk is about 20 million people with 26 districts and 91 thanas reported VL cases in 1996. Among the affected districts, Pabna, Mymensingh, Tangail, Jamalpur, Gazipur, Sirajganj, Natore, Manikganj, Dinajpur and Rajbari are the worst affected (Sarker *et al.,* 1997). An incidence rate of one per thousand (1/1000) population in affected areas is considered at present a conservative estimate as the disease is under reported and continuously spreading to areas previously free from the disease (Sarker *et al.,* 1997).

2.4 Reservoirs:

Broadly speaking, there are two types of VL, namely zoonotic VL and anthroponotic VL. In the anthroponotic form, humans act as reservoir and in the zoonotic form animals are the reservoir (WHO, 1990).

2.4.1 Human: In the anthropoetic form, which is prevalent in India, Bangladesh and some countries of East Africa, humans are directly involved as

reservoir (WHO, 1990). All efforts to find a zoonotic reservoir in Indian VL have so far been a failure (Srivastava *et al.*, 1984). Since PKDL may persist for up to 20 years such patients may act as a chronic reservoir of infection (Chulay, 1991) and an outbreak of VL has been traced to case of PKDL.

2.4.2 Dogs: In the zoonotic form, dogs are the principal reservoir in Europe and Africa around the Mediterrenean regions. Dogs are also important reservoirs in China and south and Central America.

2.4.3 Wild canines: In southern France and central Italy, foxes with in apparent infection are the reservoir. Foxes are also reservoir in Brazil and jackals are probably an important source of sporadic mainly rural cases that occur in the Middle East and Central Asia.

2.4.4 Rodents and others: Rattus rattus, the common peri-domestic rat in many countries, has been found to be infected with various Leishmania species in both Old World and New World. While its role in the maintenance of parasite populations has not been fully established, it is strongly suspected as a secondary reservoir host of *L. infantum* in Italy. *L. donovani* has been isolated from *Arvicanthis nilotica* and other rodents in Sudan and rodents are probably important in maintaining enzoonotic foci in interepidemic period (WHO, 1990).

2.5 Vectors:

Sand fly species and subspecies of *Phlebotomus* in the Old World (Asia, Africa and Europe) and *Lutzomyia* in the New World (Central and South America) are the only proven vectors of Leishmania (WHO, 1990).

In the Central and South America, *Lutzomyia longipalpis* is the main vector for transmission of VL. In the Mediterrean region, *Phlebotomus pernicious chinensis* and *Phlebotomus alexandri* are the proven vectors. In East Africa including Sudan, *Phlebotomus martini* and *Phlebotomus orientalis* and considered as vectors for the disease. In India, for the anthroponodic form of VL the proven vector is *Phlebotomus argentipes*. Besides, many other species have been implicated as vectors of leishmaniasis (WHO, 1990; Swaminathan *et al.*, 1942).

In Bangladesh, during an outbreak of kala-azar at Kalihati thana of Tangail districts sandfly species of *Phlebotomus argentipes, sergentomyia babu babu, Sergentomyia barrudi* and *Sergentomyia shortii* were identified (Masum *et al.*, 1990a). The sand flies collected from Thakurgaon district of Bangladesh during an outbreak of VL were identified as *Phlebotomus argfentipes, Phlebotomus papatasi, Sergentomyia babu babu and Sergentomyia barrudi* (Masum *et al.*, 1990b).

An entomological study on vector of kala-azar in Shahjadpur thana of Sirajgonj district showed that the species were *Phlebotomus argentipes, Phlebotomus malabaricus* and *Phlebotomus minutus*. Among these *Phlebotomus argentipes* was 70.6% (Ahmed *et al.*, 1983).

2.6 Aetiology:

2.6.1 Causative Agents

Seven complex consisting of 17 leishmania species i.e. *Leishmania donovani* complex *Leishmania tropica* complex, Leishmania major complex, *Leishmania aethiopica complex, Leishmania mexicana complex, Leishmania braziliensis complex* and *Leishmania guyanensis* complex have been identified as causative agents of leishmaniasis all over the world (WHO, 1990).

Visceral leishmaniasis is caused by parasite species of the *Leishmania donovani* complex that include *Leishmania donovani, Leishmania donovani infantum* and *Leishmania donovani chagasi* (in this thesis the three species has been referred to as *L. donovani, L. infantum* and *L. chagasi* respectively). Some undefined or unspecified species belonging to the *L. donovani* complex has been isolated from VL patients in Kenya, Ethiopia and Somalia.

Besides these species mentioned above, other species generally associated with cuteneous leishmaniasis have been isolated from VL patients in different regions of the world. Sacks *et al.*, (1995) isolated *L. tropica* from patient with

classical India VL. L. major has been isolated from a patient of VL in Israel. VL caused by *L. amazoniensis* has been reported from Bahia state in Brazil. Viscertropic syndrome caused by *L. tropica* affected a small number of American troops during Operation Desert Storm (WHO, 1990).

Old World, anthroponotic visceral leishmaniasis is caused by *L. donovani.* In India, Bangladesh, Nepal, parts of China and East Africa VL is believed to be caused by *L. donovani* other species has been identified as mentioned above.

Zoonotic visceral leishmaniasis in Mediterrenean region, China, Middle East and parts of Sub-Shara Africa is caused by *L. infantum* with dog being the principal reservoir.

New World zoonotic visceral leishmaniasis is caused by *L. chagasi* and *L. infantum* here as well, dogs are the main reservoir.

2.7 Morphological forms:

Leishmania are digenetic (existing in two forms) protozoa which exists as:

i. Amastigote form and

ii. Promastigote form.

2.7.1 Staining characteristics: Romanowsky dyes (e.g. Giemsa) stains chromatin of the nucleus and nucleic acid containing kinetoplast a brilliant red or violet, whereas the cytoplasm is stained pale blue (Neva *et al.*, 1990).

With Leishman's stain, the cytoplasm appears blue, the nucleus pink or violet and the kinetoplast, bright red (Chatterjee, 1982).

i. **Amastigote form:** Amastigotes are aflagellar, obligate intracellular form which reside within mononuclear phagocytes of their vertebrate hosts including man. Amastigotes appear as round or oval bodies ranging from 2-3 μm in major diameter. The size of amastigote from different species is known to vary. The cytoplasm of the amastigotes often stains the same as the host cell cytoplasm and only the nucleus and kinetoplast can be distinguished. The nucleus of the parasite occupies a central position or along the side of the cell membrane. The kinetoplast lies adjacent to the nucleus either tangentially or right angle to it. The kinetoplast stains more densely than the nucleus and it is variable in shape being round, oval, rod-shaped or curved in profile (Chatterjee, 1982; Neva *et al.*, 1990).

ii. **Promastigote form:** Promastigotes are flagellated extracellular form, which are found in the gut of sandflies and in *in-vitro* culture. The flagellar promastigote form measures 10-20 μm in length not including the length of the flagellum, which may equal the body length. The pale blue staining cytoplasm contains a centrally placed nucleus. The kinetoplast lies about 2 μm from

the anterior end and the flagellum emerges anteriorly. The overall shape is that of a spindle with the posterior end gradually tapering to a point (Neva *et al.,* 1990).

2.8 Life Cycle:

2.8.1 In sand fly host: The amastigotes are ingested with the first blood meal of the female sand fly in which they become transformed almost immediately into promastigotes, which multiply in the mid gut and then migrate forwards to the anterior part of the thoracic mid gut or '*cardia*'. From here they move forward to contaminate the mouthparts to be regurgitated into the wound caused by the bite at the second blood meal. In all cases, the infections are transmitted by bite. Development in the sand fly from amastigote to infective promastigote stage (metacyclic promastigotes) varies from 5-10 days (Manson-Bahr *et al.,* 1991).

2.8.2 In mammalian host: After inoculation by the sand fly, either into a capillary or the dermal tissue, the promastigotes encounter macrophages, which actively search them out and phagocytose them by receptor-mediated enocytosis. The promastigote changes into amastigote in a phagolysosome where it multiplies by binary fission. Multiplication goes on continuously till the cell becomes packed with parasites. The host cell is thereby enlarged and eventually ruptures or the amastigotes leave the macrophages by penetrating the cell membrane. The amastigotes thus released into the circulation are again

either taken up by, or invade fresh macrophages and the cycle is repeated. In this way to entire reticuloendothelial system becomes progressively infected. In the blood stream, some of the free amastigote are phagocytosed by the neutrophilic granulocytes and monocytes (macrophages). A blood-sucking sand fly draws these free amastigote forms as well as those within the monocytes during its blood-meal (Chatterjee, 1982; Manson–Bahr *et al.,* 1991).

2.9 Mode of Transmission:

The most important mode of transmission of VL is by the bite of infected female sand flies of the genera *Phlebotomus* and *lutzomyia.* A sandfly is infective to a new host from 5 to 10 days after infective blood meal. It remains infected for the rest of its life and capable of infecting several persons (Manson-Bahr *et al.,* 1991).

Transmission of VL may take place by contamination of bite wound or by contact when the insect is crushed during the time of feeding. Transmission by blood transfusion has been reported. Rarely, congenital infection from infected mother can occur. Direct transmission by sexual contact has been described. Accidental inoculation of parasites during laboratory work may results in leishmaniasis (Manson-Bahr *et al.,* 1991).

2.10 Molecular determinants of virulence of leishmania:

2.10.1 Surface Lipophosphoglycan (LPG): Lipophosphoglycan (LPG) is heterogeneous molecules and constitutes the most abundant surface component; each cell has more than 10 copies. Lipophosphoglycan is present in both leishmanial stages and is released as "excretory factors". The molecular structure of LPG consists of three portions: terminal repeats of phophorylated saccharides, a phosphorylated heptasaccharide core and lyso-alkyl-phosphotidylinositol (Turco *et al.,* 1987). The terminal saccharide-repeating units are also structurally unique and vary with Leishmania species. The difference in this portion of the LPG distinguishes anti-genetically different Leishmania species. Antigenic heterogeneity is further contributed to, probably even more significantly, by another group of relates surface inositol glycolipids. The tissue tropism of different Leishmania species may be related to the variations in their surface flycolipids (Handman *et al.,* 1987).

LPG and related glycolipids play important biological roles in Leishmania macrophage interactions. The receptor for LPG appears to be complement receptor (CR) 3 and p150, 95 (CR4) (Chang *et al.,* 1990). In addition LPG enhances the survival of promastigotes in macrophages (Handman *et al.,* 1986). Experimental evidences suggests that the mechanism of this protection may be based on the action of LPG to scavenge oxygen free radicals (Chan *et al.,* 1989) and /or to inhibit relevant enzymes, e.g. lysosomal glycosidase and protein kinase C. Other proposed biological action of LPG includes inhibition

of lymphoproliferative response and activation of T suppressor cells (Change *et al.,* 1990). Leishmanial virulence has been related to quantitative and qualitative changes of promastigote LPG.

The Leishmania LPG and related glycolipids are highly immunogenic owing to their unique structural features (Tolson *et al.,* 1989). They have long been exploited as excretory factors for serotyping of Leishmania species (Turco, 1988).

2.10.2 Glycoprotein (gp) 63: Surface glycoprotein known as gp63 was initially recognized as a major surface antigen of promastigotes by using monoclonal antibodies and surface radio iodination of living cells (Change *et al.,* 1990). It constitutes about 1% of the total cellular proteins or 500,000 copies per cell (Bordier, 1987; Bouvier *et al.,* 1985). The glycoprotein63 protein is seen to migrate on SDS polyacrylamide gels between 60-65 kD depending on the species of Leishmania (Wilson *et al.,* 1989) and which is the major antigenic protein of most promastigotes (Colomer-Gould *et al.,* 1985). glycoprotein63 has been found in all major pathogenic Leishmania species (Bourvier *et al.,* 1987), and in both stages endowed with proteolytic activity. Gp63 has been shown to cleave C3 into C3b and other C3 products (Colomer-Gould *et al.,* 1985).

By virtue of its proteolytic activity, universal presence, abundance and surface localization, gp63 is thought to be Leishmania virulence factor. The abundance of gp63 is often correlated with infectivity (Kweider *et al.*, 1989; Wilson *et al.*, 1989) and host-parasite interactions (Russell *et al.*, 1986; Wilson *et al.*, 1988). Because of gp63's proteolytic activity, it may function in the CR-mediated endocytosis of promastigote (Da Silva *et al.*, 1989) and protection of Leishmania from intralysosomal microbicidal factors (Change *et al.*, 1990). Leishmania gp63 is immunogenic; anti-gp63 antibodies have been reported in sera from patients with leishmaniasis (Heath *et al.*, 1987; Reed *et al.*, 1987). Gp63 is potentially useful antigens for immunodiagnosis (Reed *et al.*, 1987) and immunoprophylaxis (Yang *et al.*, 1990).

2.10.3 Acid Phosphatases (ACP): The cell surface acid phophatases (ACP) was initially discovered on the promastigotes stage using ultrastructure cytochemistry. Two forms of ACP namely, membrane-bound ACP and secretory ACP, are present. The two forms are antigenically distinct and each probably exist as multiple isoenzymes present in both stages of most Leishmania species. One form of the membrane-bound ACP and the secretary ACP have been purified from *L. donovani* and found to be homodimers of 120 and 134 kD. All these different forms of ACP are nonspecific monoesterase capable of hydrolyzing a variety of phosphorylated substrates (Chang *et al.*, 1990).

The membrane-bound ACP purified from promastigotes reduces the respiratory burst of neutrophils (Remaley *et al.*, 1985) and is itself resistant to oxidative metabolites (Saha *et al.*, 1985). In addition, it has been shown to dephosphorylate certain phospholipids and phosphoproteins. Thus, the ectoenzyme is thought to protect Leishmania species by interfering with the regulatory mechanism of the macrophages that produces microbial free radicals (Glew *et al.*, 1988). Its production in large quantity elicits humoral immune response of the host and may conceivably contribute to the pathobiology in leishmaniasis (Chang *et al.*, 1990).

2.10.4 Nucleotidases: 5' nucleotidase and 3' nucleotidase/nuclease have been reported to exist on the surface of some trypanosomatid protozoa including Leishmania species. The 5' nucleotidase has an electrophoretic mobility of about 70 kD on SDS-PAGE and is active in degrading both ribo and deoxyyribonucleotides. The 3' nucleotides/nuclease of 43 kD is more abundant. The latter enzyme is most active with 3' AMP and prefers RNA to DNA as substrate. The alkaline pH optima of both enzymes suggest that they probably serve such functions for promastigotes in the sandfly gut better than for amastigotes in the phagolysosomes of the macrophages (Chang *et al.*, 1990).

2.10.5 Transporters: The plasma membrane of Leishmania has been shown biochemically and genetically to possess the transport systems for folate, glucose, nucleosides, proline and ribose. The also possess a cat-ion or proton-transporting ATPase, which is apparently crucial for the homeostasis of Leishmania species and the transport of nutrients necessary for their adaptation to the changing environment in their life cycle (Chang *et al.,* 1990).

2.10.6 Cysteine Proteinases and Megasomes: The cysteine proteinase, a conserved and widespread enzyme, has been found in leishmania species generally associated with cutaneous form of leishmaniasis of South American origin. It is present in an unusual organelle, or megasome-a modified lysosome, which is noticeable in the promastigotes grown to stationary phase and becomes fully developed as they differentiate into amastigotes. This enzyme is proposed to serve a degenerative role, possibly for the nutritional benefits of the amastigotes and for releasing ammonia or other amines to modulate the host lysosomal activity for the parasites' intracellular survival and is functionally important to amastigotes (Chang *et al.,* 1990).

2.10.7 Heat-Shock Proteins: Exposure of promastigotes to elevated temperatures results in an over-expression of the classic heat-shock genes and other genes. Multiple protein bands emerge shortly after heat-shock and their number varies with different species. Only one of these proteins has been positively identified immunologically as equivalent to H sp 70 protein. Most

intriguing is the increase in the virulence seen with briefly heat-shocked promastigotes. What molecular changes in these promastigotes account for virulence remains uncertain (Chang *et al.,* 1990).

2.11 Pathogenesis:

Despite the wide range of variation of the geographical distribution, clinical manifestations and species involved, leishmanial infection shares a common feature, namely the prasitization of the phagocytic cells of major organs of the reticuloendothelial (RE) system such as spleen, liver, blood, bone marrow and skin (WHO, 1990).

With the bites of infected sand fly, the metacyclic (infective) forms of parasite are inoculated into the micro wound of the skin. Here the promastigotes are exposed to IgG and IgM, which opsonize them and are killed by activation of the membrane attack complex of complement through the classical pathway (Pearson *et al.*, 1980). Those escaping the lethal effect of serum bind to macrophages. Lipophosphoglycan (LGP) and gp63 has been considered parasite ligands (Handman *et al.*, 1985; Rizvi *et al.*, 1988; Russell *et al.*, 1988), with complement receptor (CR) 1 and CR3 and mannose-fructose receptor as the corresponding macrophage receptors (Blackwell *et al.*, 1985; Mosser *et al.*, 1985; Da Silva *et al.*, 1989). After receptor-mediated endocytosis the parasite resides within the phagolysosomes, thus are sheltered from the body's immune system. Side by side, they overcome the microbicidal conditions of the

macrophage phagolysosomes. Amastigotes and metacyclic promastigotes appear to evade oxygen-dependent destruction by triggering a minimal respiratory burst during infection due to the use of C3 receptors for internalization (Da Silva *et al.,* 1989). LPG scavenges oxygen free radicals (Chan *et al.,* 1989) and inhibits relevant enzymes e.g. lysosomal glycosidases and protein kinas C that may enhance the survival of promastigotes in macrophages (Chang *et al.,* 1990). Glycoprotein63, a parasite ectoenzyme, has been shown to protect lipid-protein substrates from intralysosomal degradation within macrophages. Protection against low pH that exists within the phagolysosmes, is accomplished by the action of a membrane protontranslocating ATPase which is located on the cytoplasmic side of the parasite surface membrane and acts by coupling ATP hydrolysis to proton-pumping activity (Chang *et al.,* 1990).

The parasites multiply by binary fission within the phagolysosomes. The infected macrophages secrete colony-stimulating factors, which stimulate precursor cells of macrophages thereby providing new target cells for the parasites, and form a granuloma with epitheliod cells and giant's cells in the dermatotropic species and hyperplasia of the reticuloendothelial cells in the viscerotropic species (WHO, 1990). The next event depends on the host resistance and partly upon toxicity and immunogenicity of the parasites. In visceral leishmaniasis, the major host response is cellular and the amastigotes in macrophages are killed due to increased production of oxygen and nitric

oxide metabolites in the macrophages (Liew *et al.,* 1990) stimulated by lymphokines particularly interferon-y (IFN-y), from activated T-helper 1 cells generated during the immune response. The released amastigotes are destroyed extracellularly with the appearance of delayed type hypersensitivity. So, in majority cases, the infections are mild and self-limiting (WHO, 1990). A small fraction of individuals who develop specific suppression of cell-mediated immunity permits the dissemination and uncontrolled multiplication of parasites leading to disease and complications.

The fundamental principle of the immunoregulation of leishmaniasis is that the parasite, which replicates in quiescent macrophages, is killed by activated macrophages. Murine models of L. major disease exemplify the th1/Th2 paradigm, in which the outcome of disease is determined by the nature and magnitude of the T-cell and cytokine responses early in infection. In infected inbred mice, production of interferon gamma by Th1 and natural killers cells mediates resistance, whereas expansion of interleukin-4-producing Th2 cells confers susceptibility (Reed *et al.,* 1993). Interleukin 12, which is an effective adjuvant in experimental vaccination against and treatment of L major infection, has a key role in the development of cell mediated immunity by inducing native T cells to differentiate into th1 cells and by inducing T cells and natural killer cells to produce interferon gamma (Afonso *et al.,* 1994). Vaccination with the DNA that encodes the LACK antigen (i.e. the leishmania homologue of receptors for activated C kinase), an immunodominant L major

antigen, induces in interleukin-12-mediated, protective Th1 response (Gurunathan *et al.*, 1997).

Not surprisingly, the T-cell and cytokine responses in infected human beings are more complex and less polarized than they are in mice, and the immune responses differ among the leishmanial syndromes and species (Berman *et al.*, 1988). Nonetheless, interferon gamma seems to be important for cure of human disease, making interleukin 12 an attractive potential adjuvant for vaccination and therapy. The presence of interleukin 10 seems to be associated with the disease process in visceral leishmaniasis (Kenney *et al.*, 1998), interleukin 4 may also contribute to disease progression (Sundar *et al.*, 1997). Although the genetic mechanisms involved in immunoregulation are also more complex in human beings than in mice, genetic susceptibility to different forms of leishmaniasis may exist (Cabrera *et al.*, 1995; Alcais *et al.*, 1997).

The genus Leishmania includes species that are the causative agents of visceral, cutaneous and mucocutaneous leishmaniasis. Infections caused by these organisms are common in both the old and new worlds, where they represent a major public health problem. As a result, Leishmania species have been the focus of much research effort aimed at the development of vaccines and novel chemotherapeutic and diagnostic reagents. The application of recombinant DNA techniques is now widespread.

Leishmania species share many common features with other trypanosomatids (e.g. *Trypanosoma brucei, Trypanosoma cruzi*) including aspects of gene expression and genome organization (Clayton *et al.*, 1988). The methods described here for the isolation of DNA and RNA from Leishmania may be applied, almost unaltered, to these other organisms. The techniques are straightforward and give reproducible and reliable results.

The Leishmania genome size has been estimated to in the region of $5x10^7$ base pairs (or 0.2 pg of DNA/cell) (Leon *et al.*, 1978), although there are variations between species (Villalba *et al.*, 1982). Different Leishmania species exhibit heterogeneity in both the number and size of their chromosomes there can be between 20 and 30 chromosomes/cell ranging in size from <200 kb to >3000 kb. Up to 25% of the genomic DNA-is made up of various forms of repetitive sequences (Leon *et al.*, 1978).

As with other trypanosomatids, approx 20% of the total cellular DNA in Leishmania is kinetoplast DNA (kDNA). There are two types, minicircle KDNA (average size 800 bp) of which there are up to 10,000 copies/cell, and maxicircle kDNA (average size 30 kb) of which there are 20-30 copies/cell. The maxicircle kDNA is analogous to mitochondrial DNA in other eukaryotes. Both maxi and minicircle DNA can encode guide RNAs (gRNA), which mediate the process of RNA editing (Simpson *et al.*, 1989 & Sturm *et al.*, 1990)

2.12 Immuneresponse in Kala -Azar:

The characteristics and functions of Th1/Th2 cell subsets have been subject of much review. Th1 cells mediate delayed type hypersensitivity (DTH) reaction while Th2 cells cannot. Both Th1 and Th2 cells can provide help for antibody production, however Th2 cells are more efficient, providing both antigen-specific and non-specific help in vitro. Th1 cells provide help in antigen-specific secondary responses with primed B cell population. However a major difference in help provided by Th1 and Th2 cells for antibody production is their ability to stimulate different antibody isotypes. Th1 clones induced more IgG2a whereas Th2 clones induce production of IgE and IgG1. The selective induction of antibody isotype appearance to be due to cytokines secreted by each cell subset. IL-4 from Th2 cell enhances IgE production; in contrast IFN-γ could switch the isotype from IgE to IgG2a. Therefore IFN-γ and IL-4 can reciprocally regulate B cell immunoglobulin production (Snapper *et. al.*, 1987). The Th1 and Th2 cells also regulate each other's induction and proliferation. IFN-γ inhibits the proliferation of Th2 cells to IL=2 and IL-4 but has no effect on Th1 cells (Gajewski *et. al.*, 1998). Th2 cells secrete IL-10 that inhibit synthesis of cytokines by Th1 cells and indirectly reduce Th1 cell proliferation (Fiorention *et al,* 1989). Th1 and Th2 cells also appear to be affected by the type of antigen presenting cell in that Th1 cells are preferentially stimulated by macrophages, whereas Th2 cells are preferentially stimulated by B cells (Gajewski *et al,* 1991).

Although initially Th1 and Th2 cells were differentiated on basis of production of different cytokine profiles, recently it has been shown that these subsets also exhibit the preferential expression of some activation markers. For example, CD30 (a member of the TNF receptor family) is mainly expressed in Th2 cells both *in-vitro* and *in-vivo*, whereas lymphocyte activation gene 3 activation gene 3 (LAG-3) a member of the immunoglobulin super family preferentially associates with Th1-like cells (Romagnani, 1997).

The factors responsible for polarization of specific immune response into a predominant Th1 or Th2 profile have been extensively investigated in mice and humans. Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineages, but rather develop from the same Th-cell precursor under the influence of environmental and genetic factors acting at the level of antigen presentation. Among the environmental factors, a role has been demonstrated for the route of antigen entry, the physical form of antigen, the type of adjuvant and the dose of antigen.

The environmental and genetic factors influence Th1/Th2 differentiation mainly by determining the predominance of a given cytokine in the microenvironment of the responding Th cell. The early prosesence of IL-4 is the most potent stimulus for Th2 differentiation (Swain *et al,* 1990). Whereas Il-12 and IFN-γ favors Th1 development. Based on both *in-vitro* and *in-vivo* studies the window of time available before a proecursor Th cell becomes

committed to a single effector cell phenotype has been estimated to be only a few days (48 hours). Therefore *in-vivo,* the relevant cytokine will need to be present at the site of T cell contact within hours of infection. One potential source for these cytokines is cells that form part of the innate arm of immunity. NK cells may be a source of IFN-γ required for diferentation of precursor Th cells to cells of Th1 phenotype (Scott, 1993). Under certain circumstances, the source of IL-4 may be small subset of $CD4^+NK1.1^+T$ cells capable of recognizing antigens presented in association with non-polymorphic β_2–microglobulin-associated molecule, CD1. However naïve Th cells themselves are able to produce small amount of IL-4 from their initial activation that may accumulate and reach threshold level leading to differentiation of Th cells to Th2 phenotype. Recently, it has been shown that IL-6 derived from antigen-presenting cells is able to polarize naive Th cells to effector Th2 cells by inducing the initial production of IL-4 in $CD4^+T$ cells (Rincon *et al,* 1997). Transforming growth factor-β (TGF-β) appears to suppress the development of Th1 cells and helps a Th2 response (Barral-Netto *et al,* 1992). Prostaglandin E_2 has been suggested to favor the development of Th2 response by inhibiting both the production of IL-12 and IFN-γ (Romagnani, 1997).

2.13 $CD4^+$T Helper Cells in Leishmaniasis:

Evidences has accumulated that the differential immune response observed in resistant and susceptible mice and healded and nom-healed mice was due to activation of different $CD4^+$T cell subsets. Although there is a difference between mice and humans in terms of the immune response to infection with different *leisshmania* species, several important principles have emerged. Resolution of leishmanial infection and protection against reinfection in susceptible humans and mice is governed by the expansion of *Leishmania*-specific helper T cells of the $CD4^+$Th1 cell type that produce IFN-γ. When present these cells activate macrophages to kill intracellular amastigotes (Murray *et al,* 1983; Sundar *et al,* 1994). IL-12 appears to play an imptant role in promoting the development of protective Th1 responses (Murray *et al,* 1995). TNF-α also appears to exert its leishmanicidal activity by activating macrophages, rather than acting directly on the parasite (Liew *et al,* 1990). Membrane-associated TNF- α on leishmania-specific $CD4^+$T cells may be important in sending activation signals to infected macrophages, resulting in parasite killing.

During progressive systemic infections in mice, there is expansion of $CD4^+$T cells of the Th2 type that secrete IL-4, but not INF-γ or IL-2 in response to leishmanial antigens. Il-4 suppresses the development of murine Th1 response and activation of macrophages by IFN-γ (Liew *et al,* 1989). Similar reciprocal activity of IL-4 and IFN-γ was obtained with human monocytes infected with

L. donovani (Lehn *et al,* 1989). In human with VL, IL-10 rather than IL-4 may be responsible for the suppression of potentially protective Th1 responses (Holaday *et al,* 1993) but others suggested that IL-4 may play a more prominent role than IL-10 in Indian kala-azar (Sundar *et al,* 1997).

2.14 Diagnosis of Visceral Leishmaniasis:

Diagnosis is provisionally made by clinical evaluation. The disease present with ferver, hepatospenomegaly, weitht loss, anaemia, leukopenia and skin pigmentaion. It is impossible to differentiate the disease clinically from other causes of splenomegaly with fever. At present, diagnosis is based on demonstration of parasites in aspiration of spleen (98% positive), bone marrow (54-86%) or lymph nodes (64%) (WHO, 1984) by direct microcopy or culture. Splenic aspiration is the most reliable method but a high-risk procedure due to chance of haemorrhage and shock. Bone marrow aspiration is safer than.

Splenic aspiration but it is a slow, painful and cumbersome procedure. Besides bone marrow smears are often negative in known positive cases (Latif *et al,* 1979). Though they have been invaluable in diagnosis and clinical management, the procedures require expert personnel and are not adaptable for screening for epidemiological purposes of for detecting clearly infections (Mohammad *et al,* 1985).

Due to the difficulties encountered in parasitological diagnosis, serological tests were more acceptable. The serological tests were more acceptable. The

serological tests commonly used for diagnosis of VL or kala-azar are aldehyde test (AT), complement fixation test (CFT), indirect flourescent antibody test (IFAT), enzyme-linked immunosorbent test (ELISA) and direct agglutination test (DAT).

2.14.1 Aldehyde Test (AT): VL is associated with a great rise in gammaglobulin, which is responsible for positive aldehyde test (AT). AT is simple and highly sensitive, usually positive after 3 months, but it is a non-specific test giving positive results in other diseases with hypergamma-globulinaemia (Ho *et al,* 1983; Cheesebrough, 1987). In the Indian subcontinent AT is commonly used as adiagnostic test particularly in rural areas. Where other facilities for diagnosis of VL are not available but the test has been found unreliable in the diagnosis of VL in many countries. Although it is a simple and sensitive test but it has little value in diagnosing recent cases which is required in seroepidemiological studies.

2.14.2 Complement fixation Test (CFT): Complement fixation test was introduced more than 60 years ago in the diagnosis of VL (Smith *at al,* 1984). Since then it has been as a serodiagnosis tool in different countries. Although, CFT becomes positive in the carly stage of infection (in 3 weeks) it is a noon-specific test. Sensitivity of this test varies with the source and method of extraction of antigen and also with different batches of antigen (Aikat *et al,* 1990) and false positive results is encountered. In addition, the test has shown

to be of limited value in routine laboratory diagnosis (Duxbury *et al.*, 1964) and large-scale epidemiological studies (Hommel *et al.*, 1978). Due to its complicated procedure the test can only be performed in reference laboratories (Rahman *et al.*, 1979).

2.14.3 Indirect Fluorescent Antibody Test (IFAT) and Enzyme –Linked Immunosorbent Assay (ELISA): The basic principle of IFAT and ELISA are same; the difference is only in the detection system of antigen-antibody reactions. The antigen fixed to a solid surface either glass slides (IFAT) or wells of microtitre plate (ELISA) are allowed to react with specific antibodies in patient sera. In IFAT, the antigen-antibody reaction is detected by the use of flourochrome conjugated antihuman IgG antibody and observed under fluorescence microscope. In ELISA, the antigen-antibody reaction is detected by using enzyme conjugated antihuman IgG antihuman IgG antibody with specific dye substrate; the result is read either visually or spectrophotometrically.

IFAT and ELISA is highly sensitive and specific test. IFAT was found to be 100% sensitive and 94-100% specific; ELISA was 96.5-100% sensitive and 87.3-98.6% specific (Jahan *et al.*, 1983; Harith *et al,* 1987; Srivastava, 1989; Muazzam, 1990). When African trypanosomiasis and Chaga's disease is taken

into consideration the specificity of both the test fall significantly (Harith *et al,* 1987).

2.14.4 Direct Agglutination Test (DAT): Cells and variety of microbial species can be directly agglutinated by antibody. Tests to detect specific antibody are carried out by serially titrating antisera in 2-fold dilutions in the presence of a constant amount of antigen. After a few hours of incubation, agglutination is complete and particles are examined either directly or microscopically for evidence of clumping. The results are usually expressed as a titer of antiserum, i.e., the highest dilution at which agglutination occurs (Rodgers, 1994).

Harith *et al,* (1986) described direct agglutination test (DAT) as a simple and economical test for detection of visceral leishmaniasis. They used trypsin-treated Coomassie Brilliant Blur-stained, formalin-preservedpromastigotes as antigen. They suggested that where trypanosomiasis is not a consideration, DAT have a sensitivity and specificity of 100%. Later Harith *et al,* (1988) modified the test procedure by adding citrate to antigen suspension and gelatin and 2-mecaptoethanol in the dilution buffer to improve the stability of the antigen in higher temperature and specificity of the test.

Harith *et al,* (1987) observed that the diagnostic performance of DAT was similar to the have IFAT; both being 100% sensitive and specific. In Bangladesh, Chowdhury *et al,* (1991) found the sensitivity and specificity of

DAT to be 96.6% and 97.2% respectively and supported its reliability in diagnosis of VL. El-Masum *et al,* (1995) showed that DAT was positive in 100% of parasitologically diagnosed VL patients from Bangladesh.

2.15 Limitation of Serological Test:

Serodiagnostic assay based on crude or whole cell antigens may be suitable for diagnosis of acute visceral leishmaniasis; early detection of infection using assays based on crude antigen has not been practical because of false reactions (Evans *et al,* 1990). In addition, the usefulness of the assays is limited in monitoring drug therapy and diagnosis of relapse by the persistence of high antibody titer for long period after chemo-therapy (Jahan *et al.*, 1983; Harith *et al,* 1986, 1987; Hailu 1990). In addition, in regions where trypanosomiasis and leishmaniasis are co-endemic, presence of cross-reactive antibodies has caused difficulties in diagnosis of VL.

The commonly used serodiagnostic assays either uses whole promastigotes. Therefore these tests are unable to detect antibody response to specific antigen of the organism. The antibody response is likely to vary during the disease process of kala-azar and a definite pattern of response may be seen.

2.16 Immuno0blotting of Western Blotting:

A specific technique and the term 'blotting' were first joined in 1975 when E.M. Southern described the transfer of electrophoretically separated single-stranded DNA from gels to an immobilized state on a membrane (southern,

1975). The approach was soon applied to RNA by Alwine *et al,* (1979) and in 1979, Renart *et al,* and electrophoretic transfer respectively. The concept of immobilization added a new dimension to analytic electrophoresis. Prior to this biochemical characterisation of individual proteins buried within separation support has been difficult and unsatisfactory particularly in tight such as polyacrylamide.

The majority of the drawbacks are eliminated when proteins of the separation gel are transferred to a protein binding membrane (immobilizing matrix) followed by antibody incubation. In this way analytic potential of the separation technique is expanded with the specificity and sensitivity of immunodetection. This idea was first practically employed by Towbin *et al,* (1979) who introduced the technique of electroblotting from sodium dodecyl sulphate polyacrylamide gels to nitrocellulose sheets followed by immunodetection.

The most common form of immunoblotting utilizes immobilized proteins on solid support to detect protein-specific antibodies. This technique is often referred to as Western blotting, a team derived from the mames for DNA blotting (Southern blotting after its inventor) and RNA blotting (subsequently named Northern blotting to differentiate it from DNA blotting). Protein separated by gel elctrophoresis are transferred to an immobilizing membrane usually made from nitrocellulose, polyvinylidene diflouride or nylon. Serum specimen is applied and antibody binds to protein on the support matrix. Bound

antibody is then detected by use of fluorescence-, enzyme- or radionuclide-labelled anti-immunoglobulin specific for the test serum species. Western blooting has been widely applied in basic research and has been useful clinically as a comfirmatory serological test in a variety of infectious diseases (Miller *et al.*, 1995).

The general components of immunoblotting as used for immunodiagnosis with human sera include (i) electrophoretic separation of protein antigen on gels (usually sodium dodecyl sulphate-polyacrylamide) (ii) electrophoretic transfer of the protein bands to a nitrocellulose or other support membrane (iii) blocking of free protein binding sites on the membrane, (iv) addition of test serum and (v) detection of the specifically bound serum antibodies. The results seen as bands formed are compared with bands obtained with known positive and negative sera for the specific microbial antigens used in the test.

This technique has been used in determining the specific antibody response to different polypeptides of respective causative organisms in many parasitic diseases (Steven *et at,* 1987; Santo *et al,* 1990; Hong *et al,* 1997). Immunoblot analysis is assumed to increase specificity gained by visualization of antibody reactivity with individual parasite antigen.

Western blot analysis was found to have a sensitivity of 100% and specificity of 98% in detecting VL and was more sensitive than IFAT or ELISA (Mary *et*

al, 1992). In addition, immunoblot assay provides confirmation of fales positive results obtained by other serological test (Da Costa *et al,* 1996). Berrahal *et al,* (1996) found that immunoblot is sensitive enough to detect asymptomatic infection and could be a valuable tool for studies monitoring the transmission of the disease and vaccination trials.

Many researchers around the world have used Western blot technique for detection of specific humoral immune response to leishmanial antigens in patients with leshmaniasis.

Dos Santos *et al,* (1987) using soluble antigens from *L. d. chagasi* showed that sera from himan visceral leishmaniasis reacted with 36 polypeptides with molecular weights ranging from 14,4000 to 123,000. Two polypeptides of M. 119,000 and 123,000 reacted with all the sera from visceral leishmaniasis patients.

Reed *at al,* (1987) using cloned *Leishmania donovani* chagasi promastigotes and immunoblot analysis observed that person infected with *L. chagasi* infection had antibodies to *L. chagasi* of approximately 32 to 35 kD which was not recognized by persons infected with other Leishmania species. Antigen in the 62 to 66 kD region were recognized by all individual with *L. chagasi* and L. mexicana amazonensis but were not recognized by individuals in other diseases (T. cruzi, Myco. Tuberculosis, Myco. leprae) or by control sera. This region was found to contain at least four distinct bands. 62 to 63 kD protein was eluted

and was used to diagnose *L. chagasi* infection by ELISA. They found that eluted 62-63 kD protein was 100% specific and sensitive in the diagnosis of *L. chagasi* infection whereas crude *L. chagasi* antigen gave false positive results with *T. cruzi* and mycobacterial infections.

Evans *et al,* (1989) evaluated the antibody responses in American VL by immunoblotting using *L. chagasi* antigen. Immunoblots of the patient's sera recognized multiple bands; the most frequent of which were at approximately 116, 70 and 26 kD. Less frequently observed were bands at approximately 93, 74, 62, 46 and 32 kD.

Using *L. infantum* promastigote antigen, Rolland et al, (1994) observed that sera from VL patients from Europe, Africa and South America, identified numerous polypeptide bands in immunoblots. The IgG response of the different human VL sera revealed variability of the antigen-binding patterns in blot. Eight bands were common to all VL sera, corresponding to antigens of 115, 94, 90, 75, 70, 40, 30 and 20 kD. The 94 kD control sera.

Tebourski *et al*, (1994) tested sera from patients suffering from Mediterrenean VL caused by *L. infantum*, with both membrane-bound and soluble antigens prepared from *L. infantum* parasites. With membrane-bound antigen, serum specimens reacted variably with 4 to 17 antigenic components ranging in molecular mass from 10 to 80 kD. Six immunodominant regions, namely 80,

74, 68, 56-64, 32 and <20 kD, were observed in the immunoblot. The major reactive band was a 32 kD polypeptide which was recognized by 95% of the serum specimens. Interestingly the majority (90%) of serum samples from patients of Mediterrenean VL did not react with the major surface glycoprotein g63. although the most prominent protein of membrane-bound antigen migrating as a diffuse band at 56-64 kD was identified with mouse monoclonal antibody as being the major surface glycoprotein gp63. When *L. infantum* soluble antigens were immunoblotted with sera from patients Mediterrenean VL, several reactive bands were identified; the ranged in molecular mass from 12 to 130 kD. The most intensely stained band was a doublet of 74 and 80 kD (74%). The other reactive bands were 12, 80, 45/49, 130, 23, 32 and 28 kD.

Ghosh *et al,* (1995) reported variable patterns of reactivity by kala-azar and PKDL sera with IgG class-specific reagents in Immunoblot analysis. Certain common bands around 60- and 63 kD and 28-kD regions were recognizer. The further showed that her 28-kDa bands was preferentially recognized by IgG_2 isotpe, while the 20- to 22-kDa and 60- to 63-kDa bands were recognized by the IgG_1 isotype. Antibodies belonging to the IgG_3 isotype reacted to antigens primarily in the region of 14 to 34 kD.

In this study, humoral immune response to *L donovani* polypeptides in visceral leishmaniasis patients from Bangladesh was examined by Western blot analysis.

2.17 Molecular Approach:

In recent years, PCR-based diagnostic methods have been described for leishmaniasis, with a wide range of sensitivity and specificity. An excellent target for a sensitive and rapid detection method is the kinetoplast mini-circle DNA, which is present at thousands of copies per cell. The mini-circles have been used as targets for selective amplification of parasite DNA in various studies (Nuzum *et al.,* 1995; Smyth *et al.,* 1992). The identification of conserved sequence elements represented within the kineto plast DNA (kDNA) of a given species of leishmania would allow the design of oligonucleotide primers to be used for species-specific identification of parasites in clinical samples. We have analyzed kDNA sequences from Old World leishmaniasis and designed primers specific for L. donovani species to detect kDNA from a single parasite in the presence of huge excesses of human DNA. The utility of the primers designed for L. donovani has been examined in clinical samples from patients with KA and PKDL in India. The PCR test was found to be sensitive enough to detect parasite DNA from peripheral blood of patients with KA and from skin lesions of patients with PKDL. Furthermore, the test was specific for L. donovani species of the parasite, leading to simultaneous species identification of the parasite (Salotra *et al.,* 2001).

The identification and characterization of Leishmania from parasites, as well as reservoir hosts and vectors, is important for understanding the epidemiology

and transmission of leishmaniasis. In addition, the characterization of parasites isolated from patients may affect decisions regarding drug treatment, since disease pathology is generally associated with specific leishmania species. In the Old World, cautaneous leishmaniasis (CL) is usually caused by leishmania major, *L. tropica*, or *L. aethiopica*; mucocutaneous leishmaniasis (MCL) by *L. aethiopica*; and visceral leishmaniasis (VL) by *L. donovani* or *L. infantum*. Diagnosis and parasite identification is still largely based on the microscopic observation of parasites in stained tissue or growth in culture, with isoenzyme analysis the "gold standard" for strain characterization. This latter technique, though relatively simple, is time consuming, since each isolate must be examined by multiple enzyme reactions. Other techniques for Leishmania characterization, such as monoclonal antibodies or excreted factor (EF) typing, may be affected by antigenic changes during growth in culture or differences between species isolated from geographically distant regions (Lainson *et al.*, 1987; Eisenberger *et al.*, 1997).

Recently, DNA-based technologies have been adapted for the detection and characterization of Leishmania. However, many of these techniques are still expensive and/or time consuming and even the most promising systems appear to have some drawbacks. PCR and Southern blotting with species-specific primers or probes have been used for direct diagnosis, although the latter technique appears to be less sensitive than PCR and employs radioisotopes which may be difficult to obtain in some developing regions or Old World

species and subspecies of Leishmania has been used to characterize both promastigotes grown in culture and amastigotes directly in tissue samples. This system is extremely sensitive and can detect even of parasite DNA. Primers derived from miniexon and intergenic sequences have also been used with equal success for leishmanial characterization by PCR. Polymerase chain reaction using different primers, followed by restriction enzyme digestion and Southern blot analysis of the products, has also been utilized to characterize Leishmania species. However, species-specific parasite identification, either directly by PCR or combined with southern blotting, has required the design of individual primers sets for each species and subspecies.

Some researcher developed a permissively primed intergenic polymorphic (PPIP)–PCR, which combines the best attributes of specific and AP-PCR. This method pairs a specific parasite primer taken from a unique leishmanial DNA region with a nonleishmanial oligonucleotide primer, taken from the plasmid. (Filnn *et al.*, 1992).

Researchers showed that PCR could distinguish between the different Old World Leishmania complexes: *L major, L. tropica. L. donovani*, and *L. aethiopica*. The PCR products can be analyzed directly by agarose gel electrophoresis and give simple patterns, which are unique for each complex. DNA from nonkinetoplastid sources is not amplified, suggesting that it may be

possible to use PCR for the direct diagnosis of Leishmania parasites in host tissue (Eisenberger *et al.*, 1997).

Leishmaniasis is widely distributed and endemic in 88 countries around the world. Visceral leishmaniasis (VL), or kala-azar, if untreated, is usually fatal. Even in treated patients, the fatality rate may be as high as 30% because of late or missed diagnoses. The prevalence of VL has been increasing in the hill foci of western China in recent years. Early diagnosis and efficient treatment could reduce mortality if a simple and accurate diagnostic technique was available.

Antibodies specific for Leishmania may remain in the serum long after patients are cured. Therefore, current serologic tests cannot be used to differentiate between active and cured cases, although many efforts have been attempted for this purpose and for evaluation of the efficacy of chemotherapy. Polymerase chain reaction (PCR) has several advantages over other diagnostic techniques. In addition to diagnosing VL accurately, PCR can differentiate the species or strain of the pathogen. Definitive diagnosis of VL now relies on the identification of the parasites in stained smears or culture of bone marrow, spleen, or lymph node, all of which require invasive procedures. Furthermore, the use of bone marrow for diagnosis of VL has a misdiagnosis rate of 16.5% in the hill foci of China (Hu, *et al.*, 1992).

Researchers previously obtained 3 L. donovani species-specific recombinant plasmids by inserting the Alu I fragments of kinetoplast DNA (kDNA) into the

Sma I site of plasmid pUC18. The reaction was used to detect L. donovani in the bone marrow and blood of 22 patients and also in bone marrow and spleen samples of 12 infected dogs as well as 2 control dogs (Hu, *et al.*, 2000).

The polymerase chain reaction (PCR), used to detect parasite kinetoplast-minicircle DNA, has been shown to be virtually 100% sensitive and specific in chronically infected persons. one of which (NM12) was predominant, representing 75% of the kDNA network. The NM12 minicircle class emerged as a useful target for the development of a PCR-based DNA detection system for L. donovani infections, targeting the kDNA minicircles of these parasites (Singh *et al.*, 1999).

Recently, the application of techniques based on detection of parasite deoxyribonucleic acid (DNA), such as the polymerase chain reaction (PCR) for diagnosis of cutaneous and visceral leishmaniasis has been reported. The highly sensitive PCR technique was used to amplify kinetoplast DNA minicircles or miniexon-derived ribonucleic acid genes from blood, bone marrow or spleen samples from VL patients. However, most of these patients had a previous history of VL and antileishmanial treatment; therefore, the ability of the PCR to detect early, untreated infections could not be assessed (Adhya *et al.*, 1995).

PCR of blood DNA was carried as previously described. Briefly, heparinized blood was haemolysed and DNA extracted from the leucocyte-enriched raction

by deproteinization and ethanol precipitation. The DNA was suspended in 10 uL of 10 mM Tris-HCI (pH 7.5) plus 1 mM ethylenediamineteraacetic acid. The conditions for amplification in a thermal cycler denaturation, 93 degree, 1 min; annealing, 55 degree, 1 min; extension, 72 degree, 1 min; 35 cycles; final extension for 10 min at 72 degree. PCR products were detected by gel electrophoresis followed by ethidium bromide staining. Samples yielding a specific 180 base pair (bp) product were considered positive (Adhya *et al.*, 1995).

The fundamental principle of the immunoregulation of leishmaniasis is that the parasite, which replicates in quiescent macrophages, is killed by activated macrophages. Murine models of L major disease exemplify the Th1/Th2 paradigm, in which the outcome of disease is determined by the nature and magnitude of the T-cell and cytokine responses early in infection. In infected in bred mice, production of interferon gamma by th1 and natural killers' cells mediates resistance, whereas expansion of interleukin-4-producing Th2 cells confers susceptibility. Interleukin 12, which is an effective adjuvant in experimental vaccination against and treatment of L major infection, has a key role in the development of cell-mediated immunity by inducing native T cells to differentiate into Th1 cells and by inducing T cells and natural killer cells to produce encodes the LACK antigen (i.e., the leishmania homologue of receptors for activated C kinase), an immunodominant L major antigen, induces in interleukin-12-mediated, protective Th1 response.

CHAPTER III

MATERIALS AND METHODS

3. Materials and Methods

The study was designed for two groups of population, one group purposively collected for immunological and molecular studies. These patients were inclued from the referred confirmed KA cases. Individuals who clinically presented with the features of KA, blood samples were positive for ICT test and bone marrow were positive for LD bodies were included in the study. Another group was included from sero-epidemiological study conducted in an endemic northeastern district of Bangladesh, which is about 200 kilometer from Dhaka. The study area is Fulbaria upazilla that is situated in north south–west part of Mymensing district. This is a rural area with a population of 4,28,998.

3.1 Study period and place:

The samples were collected in the months of December 2001 to June 2004. All the experiments were carried out at the Department of Microbiology, BIRDEM and NIPSOM.

3.2 Study population:

The specimens were collected from the patients who were included in the study when they have shown positive clinical symptoms and became positive by ICT test for kala-azar from Fulbaria upazilla of Mymensingh district and Gazipur upazilla under Gazipur district. For sero-surveillance Fulbaria upazilla was selected purposively. Ten villages of Fulbaria were included and from each village 180 respondents were taken by systematic random sampling.

3.3 Definition of kala-azar:

Diagnosis of KA l is based on typical clinical features and demonstration of LD bodies and positive parasite culture. Parasitization of the macrophages especially of the liver, spleen, and bone marrow. Clinically characterized by fever, splenomegaly, hepatomegaly, pancytopenia and immunological unresponsiveness and fatal if not treated.

3.4 Definition of cure:

A cure is defined as return of the temperature to normal, a decrease in the size of the spleen and absence of parasite in the bone marrow smear. Negative bone marrow or spleen aspirates 2 weeks after the end of treatment. Treatment regime for visceral leismmanias is with pentavalent antimony (Urea Stibamine Gluconate) given 20 mg/kg once a day for 21-40 days (intramuscularly or intravenously).

3.5 Location of the area and villages:

3.5.1 Study population: For immunological and molecular study, among the seropositive cases in the study 131 were active KA patients where 70 patients were from serosurvillence and 61 were from referred cases. The specimens were collected when they have shown positive clinical symptoms and became positive by ICT test for kala-azar and LD body positive for bone marrow from Fulbaria upazilla of Mymensingh district and Gazipur upazilla under Gazipur district. For sero-surveillance, Fulbaria upazilla was selected purposively. Ten

villages of Fulbaria were included and from each village 180 respondents were taken by systematic random sampling. Mymensing district was selected purposively for sero-epidemiology of KA as this district had a high prevalence of kala-azar. Multistage sampling method was followed for the selection of the population. Upazilla was selected purposively. Total union was thirteen from which ten were selected by systematic random sampling. One village was selected from each union. A map of the Fulbaria upazilla with position of the villages is attached in appendix.

3.5.2 Sample selection: Equal number of samples was collected from each village. Samples were collected systematically selecting households at a defined interval depending on the total number of population of that village.

3.6 Data collection procedure:

A questionnaire, both open and close ended was used for collecting data and pertinent information by face-to-face interview. Data on age sex, clinical features, socio-economic status, religion, education, housing condition, use of mosquito net, condition of the house were noted on questionnaire.

3.6.1 Blood collection: Blood samples were collected aseptically from the study population. The serum was separated immediately for diagnosis by ICT/ELISA method and rests were being kept in the storage at–20 degree centigrade for antibody analysis. Sero-positive VL patients would be examined their clinical findings and other related information would be recorded. The

patients were also treated in the hospital. Relapse cases were given further treatment. Collection was also done 15 days, 3 months, 6 months, 9 months and 2 years after treatment. In the present study a total of 131 Kala-azar patients were included. The spacimen of bone marrow and blood were collection. For sero-surveillance, 1844 blood samples were collected from Fulbaria upazilla of Mymensingh district. From blood hemoglobin TC, DC, ESR, BT, CT, estimation of total IgG and its subclasses were performed and buffy coat were separated for culture and PCR analysis.

3.6.2 Bone marrow aspiration, slide preparation, staining and culture: Bone marrow aspiration was performed after using standard technique and under direct supervision of one of the author. An aliquot of aspirated bone marrow was stained with Giemsa stain and another was cultured in McNeal, Novy, Nicolle medium and another two aliquot one in normal saline and another in formalin are preserved at -20^0 for PCR analysis. Slides were prepared from bone marrow and stained by both Leisman and Ziemsa stain. After washing with tap water. Slide was dried and examined under microscope. Bone marrow aspirates was delivered onto a clean glass slide, about 1 cm from one end and then most of the blood was sucked off quickly with a fine pipette onto the edge of each drop. Then a film of 3-5 cm in length of bone fragments and remaining blood was made using a smooth edge glass spreader of not more than 2 cm in width. The slide was then allowed to dry and stained with

Leishmam's stain and examinred under microscope for the presence of *Leishmania donovani*.

With strict aseptic precautions the bone marrow aspirates was inoculated into the liquid phase of NNN media and incubated at 230c for 7 days. After 7 days, using 3mm sterile wire loop, a drop of fluid was transferred from the culture tube on a slide and examined under microscope for the presence of promastigote. If no parasites were seen, the cultures were re-incubated and reexamined weekly for up to 4-6 weeks. If positive, cultures were maintained by sub culturing in NNN media and also cryopreserved stabilities were stored in liquid nitrogen.

3.6.3 Cryopreservation of leishmania parasites as stabilate (Frozen Promastigotes): Cryopreservation of leishmania parasites was performed as per method described by Evans (1989) and WHO (1990). Leishmania parasites were cultured in monophasic Eagle's minimum essential media containing 20% foetal calf serum, sodium essential media containing 20% foetal calf serum, sodium bicarbonate 2.2 mg/ml, penicillin 200 IU/ml, streptomycin 100 µg/ml, penicillin 200 IU/ml, streptomycin 100 µg/ml, pH 7.2 (Appendix 5). This medium was henceforth called as Complete Eagle's Minimum Essential Media (CEMEM). When a minimum promastigote concentrate of 10^6 per ml was obtained, the parasites were processed for cryopreservation in the following manner.

a. With aseptic precautions, 10 ml of culture containing growth of actively motile promastigotes with a concentration of 10^6 per ml of media was transferred into a sterile glass tube, kept on ice.

b. Sterile dimethyl sulphoxide (DMSO), used as cryoprotectant was added to culture to give a concentration of 7.5% of dimethyl sulphoxide (DMSO) in the final volume and mixed thoroughly.

c. The cryoprotected samples were transferred to the sterile labelled screw capped plastic cryovials of 2 ml capacity, filled upto $2/3^{rd}$ and frozen in the following manner.

d. The samples were then cooled to 4^0 C in a refrigerator and kept at this temperature for a minimum of 1 hour.

e. It was then transferred to a freezer maintained at -20^0 C and left for 24 hours, then transferred to another freezer maintained at -70^0 C for at least 24 hours and finally transferred into liquid nitrogen (-196^0 C) where the were stored.

f. The contents of one of the frozen vials of each isolate maintained at -196^0 C were thawed and cultured to check the viability of the stabilities. If no growth was obtained, a fresh stabilate was made from the original culture.

3.6.4 ICT: ICT test kit (Inbios, USA) was procured commercially. The test was performed according to the instruction of the manufacturer. Serum samples were assayed for anti-K39 IgG using the One-Step In Sure Visceral Leishmaniasis Test (InBios). (InBios, which donated the strip tests, had no role in interpreting the data or in deciding whether the study was to be submitted for publication). The test-strip membrane is coated on the bottom with a band of recombinant K39 antigen and on the top with immobilized anti-protein. An antibody to detect IgG; and protein A-gold conjugate is used as the immuno-chromatographic detection reagent. One drop of serum was placed on the absorbent pad on the strip bottom, 3 drops of test buffer were added to the pad, and the mixture was allowed to migrate up the strip by capillary action. After 2-5 min, the appearance of a red upper (control) line indicated the presence of IgG and proper test functioning; a red lower line indicated the presence of anti-K39 IgG and a positive test result. Strip testing was performed before results from the rest of the baseline evaluation were available.

3.6.5 Mass Cultivation: Isolate was inoculated in 6(15 ml capacity) screw capped. Each containing 30 ml of CEMEM and incubated at 23^0 C for 3-7 days. From the 3rd day onward little amount of culture fluid containing parasite was taken out from the tubes with aseptic precautions and counted in Neubauer counting chamber. Parasites were harvested when its concentration reached above 10^6 per ml of media.

3.6.6 Harvesting promastigote cultures: Prior to the harvesting of the organisms thin Giemsa stained and Gram stained films were prepared for each population of organisms. Films were checked for aberrant morphology or evidence of cultures contaminants particularly bacteria and fungi and also subculture was done to exclude any contaminant. If no contamination was present, harvesting of promastigote was done as per method described by Evans in 1989.

a. Promastigotes grown CEMEM were centrifuged at 2000 g for 20 minutes at 4^0 C in screw capped centrifuge tubes.

b. A sterile Pasteur pipette carefully discarded supernatant and the pelleted organisms were re-suspended in about 5 ml of proline balanced salt solution (PBBS) pH 7.2 and centrifuged as before.

c. The resulting pellet was re-suspended in 1.0 ml of Proline Balanced Salt Solution (PBSS) and transferred to a 2 ml eppendorf tube and centrifuged again at 2000 g for 20 minutes at 4^0 C. The supernatant was carefully discarded as before.

d. A volume of 2 mM stabilizer solution containing *EDTA 2 mM + DTT 0.308 mg/ml + EACA 0.262 mg/ml was added to equal volume of the pelleted organism in tube and left over night at – 20^0C.

e. EDTA = Ethylene diamaine tetra acetic acid, DTT = Dithio threitol EACA = E amino caproic acid.

3.6.7 Enzyme Linked Immunosorbent Assay: The total number of people tested for ELISA was 1844 for sero-surveillance. On the other hand, 131 kala-azar confirmed patients were selected for in depth study for IgG subclass study. The control population consisted of 150 healthy individual from non-endemic areas. Pulmonary TB patient, and Widal positive patient was included in this group. Another 1539 healthy individual from endemic areas were included as control. ELISA using K-39 antigen was employed to detect anti leishmanial 1gG and subclass antibodies in the serum sample. 131 leishmania-infected persons were positive for total 1gG and 1gG subclass. Non-endemic person and healthy endemic and typhoid fever persons were negative for total 1gG and 1gG subclass. Antileishmanial 1gG1 subclass could detect positive cases with appropriate cut off values that can eliminate gross reactivates. This serological method can be a very useful tool for leishmania diagnosis and survey.

Experimental approach for determination IgG and its subclasses by ELISA technique was employed for determination of anti-leishmanial subclass specific IgG (Ghos *et al.*, 1995). Different antigenic preparation from cultured promastigotes was used for determination of anti-leishmanial IgG subclasses. Finally ELISA plates were coated with K-39 recombinant protein antigen with 25 µg/well. After washing the plates with 200 µl wash buffer 3 times. Next 250 µl of blocking Buffer was added to each well and incubated at room temperature. A positive control, negative control a dilution of 1/200 is used. For positive control 5 serial dilutions beginning from 1/100 to 1/12800 tried.

Elisa was performed for 1844 samples and positive cases were detected after detecting the cut off value from the positive and negative control.

3.6.8 In House ELISA: Plates were quoted with whole promastigote and blocked with buffer then IgG and its sub-classes were also examined from the patients' serum. The procedure of quoting and blocking is same with K39 antigen.

3.7 Western blot (Towbin et al., 1979):

3.7.1 Preparation of Antigens: Antigens were prepared according to the method described by Maizel *et al.*, (1991).

3.7.2 Parasites: Antigen was prepared from *L. donovani*, which was used for sodium dodecyl sulphate polyacrylamide gel ectrophoresis (SDS-PAGE), and subsequently for western blotting and immuno-staining. The cultured strain was maintained in the laboratory of Department of Microbiology, BIRDEM, Dhaka by weekly passage in NNN media (biphasic medium constituted by a brain-heart infusion agar enriched with human blood and overlaid with Locke's solution) and incubated at 22°C.

- Promastigotes from maintenance culture, were grown in Eagle's Minimum Essential Media (EMEM) with Balanced Serum Salt Solution (BSSS) containing 20% foetal calf serum (FCS) and antibiotics, (penicillin and streptomycin) at 22°C (Appendix III). Three drops of supernatant fluid of NNN media containing promastigotes were inoculated in 10-15 tubes

containing 10ml aliquots of EMEM. Small aliquots of culture media were used instead of mass culture as a precaution against contamination.

- After 7 days of growth, the cultures were examined for the promastigotes. The culture tubes were centrifuged at 2000 rpm for 15 minutes at 4°C. The supernatant was discarded.
- About 3 ml of cold phosphate buffered saline (PBS), pH 7.2, was added to each tube and centrifuged at 2000 rpm for 10 minutes at 4°C. the supernatant was discarded. The washing was repeated 3 times.
- The promastigotes were then harvested into one tube and cold PBS washings of the culture tubes were added. A final wash was given. After discarding the supernatant the pellet was resuspended in the remaining fluid.
- The promastigotes were then heat killed by boiling in a water bath at 100°C for 10 minutes. An equal volume of eletrophoresis sample buffer was also heated for the same period of time.
- After heating, the volume of sample buffer was added to the heat killed promastigotes. The parasites were then lysed by boiling the mixture for 5 minutes at 100°C in a water bath.
- The lysate was centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was collected and used as antigen for SDS-page: the antigen consisted of a mixture of L. donovani promastigote polypeptides.
- Three batches of antigen were prepared using the same method.

3.8 Sodium Dodecyl Sulphate Polyacrylamide gel:

3.8.1 Electrophoresis (SDS-PAGE): SDS-PAGE was done.

3.8.2 Equipments used:

1. Protean II Slab Electrophoresis Cell, 16 cm (Bio-Rad)

   It contains the following parts:

   a) Electrophoresis tank with lower buffer chamber

   b) Central cooling core with upper buffer dam

   c) Cell lid with electrical connections.

2. Gel casting stand with adjustable legs (Bio-Rad)
3. Sandwich clamps
4. Glass plates – two; sizes 180 x 160 and 180 x 180 mm
5. Spacers – two (1.5 mm thick)
6. Comb – one (1.5 mm thick)
7. Rubber gasket (20 x 200 mm)
8. Computer Controller Eletrophoresis Power Supply – 3000 Xi model (Bio-Rad)
9. Degassing Flask with rubber stopper
10. Vacuum pump
11. Syringe with needles
12. Pipettes and other laboratory apparatuses.

3.8.3 Buffers and Reagents: (Preparation: Appendix)

1. 30% Acrylamide solution
2. Lower gell (resolving gel) buffer (1.5M Tris Hcl, 0.4% SDS, pH 8.8)
3. Upper gel (stacking gel) buffer (0.5M Tris Hcl, 0.4% SDS, pH 6.8)
4. 10% Ammonium persulphate (APS)
5. N, N, N, N, Tetramethyl ethylene diamine (TEMED)
6. Sample buffer (0.625M Tris, 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.001% brophenol blue, pH 6.8).
7. Electrode (running) buffer (0.25M Tris, 0.19M glycine, 0.1% SDS, pH 8.2 – 8.5)
8. Deionised water
9. Molecular mass marker (Bio-Rad)

   The molecular mass marker consists of a mixture of prestained proteins of known molecular masses.

i.	Phosphorylase B	106 kilodaltons (kD)
ii.	Bovine serum albumin	80 kD
iii.	Ova albumin	49.5 kD
iv.	Carbonic anhydrase	32.5 kD
v.	Soyabean trypsin inhibitor	27.5 kD
vi.	Lysozyme	18.5 kD

3.8.4 Procedure:

1. The glass plates, buffer chambers, comb and spacers were cleaned with laboratory detergents and rinsed with distilled water. The glass plates were allowed to dry in a vertical position.
2. The gel-casting stand was made perfectly horizontal by adjusting the legs and using the leveling bubble. The rubber strip was placed flat on the stand.
3. Placing the two spacer between the two glass plates, one on each side, then assembled the glass plate sandwich. The sandwich clamps were slipped on both sides of the glass plates sandwich. The whole assembly was then transferred into the rubber strip on the casting stand. The glass plates were kept vertical. The knobs of the clamps were tightened and final locked in position with the clamps.
4. About 10 ml of deionised water was poured inside the sandwich and the level of water was marked. The level of water was checked after 10 minutes for any sign of leakage. The water was removed before casting the gel.
5. Gels were prepared according to the recipe given in the table below. 12% and 4% polyacrylamide gel were used as lower and upper gel respectively. To make 12% lowers gell for one slab, acrylamide solution, lower gel buffer and deionised water were taken in a degassing flask and mixed. The solution was then degassed with the help of a

vacuum pump. APS and TEMED were added. The solution was then pipetted into the slab space to a height of about 10.5 cm. Five milliliter of deionised water was overlayed carefully on the top of the gel with a steady even rate of delivery so that the surface of the monomer solution was not disturbed and solution was left overnight for polymerisation.

6. After polymerisation the overlay was removed. A 4% acrylamide upper gel monomer solution was prepared by mixing acrylamide solution, upper gel buffer, deionised water, APS and TEMED. The solution was immediately pipette on to of the lower gel. The comb was placed between the plates carefully so that no air bubbles were trapped under the comb teeth. After 30 minutes. When the upper gel had polymerized the comb was removed slowly pulling straight up to avoid tearing the gel. The wells were rinsed with running buffer using a syringe and needle to remove unpolymerised monomer and any small fragments of polyacrylamide.

7. A volume of antigen containing 180 μg of protein (about 90 μl) was taken in an Eppendorf tube and heated in a water bath at 100°C for 3 minutes. Ten microlitre of prestained molecular mass markers were loaded in one of the well. Two micro liter of preheated antigen (20 μg of protein) was loaded carefully into each of the empty well. Then all the wells were carefully filled with running buffer.

8. The gel glass plate sandwich was carefully removed from the casting stand but the clamps were kept in place. The whole assembly was then slipped along one side of the central cooling core and locked in position. The other side was balanced. The cooling core with upper buffer dam with the attached glass plate assembly was placed in the electrophoresis tank filled with about 2 L of running buffer. About 0.5 L of running buffer was poured slowly into the upper chamber so as not to disturb the samples layered in the wells. Any air bubbles trapped under the lower surface of the glass plate sandwich were removed. The lid with the electrical connections was put on properly.
9. Finally the cell was connected to the power pack and the gel was run at a constant current of 35 mA till the broophenol dye reached the lower end of the gel, about 3-3.5 hours.
10. After electrophoresis the gel sandwich was dissembled and the gel was used for electroblotting and staining.

3.8.5 Reagents:

3.8.5.1 0.2% Coomassie blue

Preparation:

Coomassie Blue R-250, (Sigma)	200 mg
Methanol (absolute)	45 ml
Glacial acetic acid	5 ml

The Coomassie blue was dissolved in the mixture of absolute methanol and acetic acid. The volume was made up to 100 ml.

3.8.5.2 Destaining solution (7% acetic acid)

Preparation:

Glacial acetic acid	140 ml
Deionised water	1860 ml

The reagents were mixed together.

3.8.5.3 Procedure:

1. The gel was placed in 0.2% Coomassie blue stain for 1 hour with constant shaking at room temperature.
2. The stain was poured off and the gel was washed well in tap water to take off the excess stain.
3. Then destaining solution was added. The tray was put in a water-bath at 80°C for rapid edestaining.
4. The destaining solution was changed a few times and destaining was continued till the background became clear.
5. The destaining solution was poured off and the gel was washed in tap water.

3.9 Immunoblot:

3.9.1 Equipment used:

1. Trans-Blot Electrophoretic Transfer Cell (Bio-Rad)

   Electrophoretic transfer cell includes –

   a) Transfer cell

   b) Gel holder

   c) Safety lid with power cables

2. Computer Controlled Electrophoresis Power Supply – 3000Xi model (Bio-Rad)

3. Nitrocellulose membranes (NCM), 0.2 µm pore size (Bio-Rad)

4. Fibre pads, 2 pieces

5. Filter papers, 3 mm, 2 pieces

6. Trays for soaking NCM and assembling the gel cassette

3.9.2. Procedure:

1. The transfer buffer was chilled before use.

2. One piece of NCM and pieces of 3-mm filter paper of same size of the resolving gel (10.5 x 16-mm) were cut. Gloves were worn while handling NCM.

3. The NCM, filter papers and the fiber pads were soaked in transfer buffer.

4. The polyacrylamide gel was seperated from the glass plates. The stacking (upper gel) was removed and the left lower hand corner was cut for orientation of sides. The gel holder was opened and placed in a tray with the smoky gray side (cathode) lying flat. A prewetted fibre pad was placed on the gray panel of the holder. A piece of saturated filter paper was placed on top of the pad. The gel was then carefully placed on the filter paper. The prewetted NCM was then placed on the gel. Care was taken so that no air bubble was trapped between the gel and the NCM. Any air bubble that might have been trapped was removed by roller-pin exclusion method using a test tube or a glass pipette. Placing a piece of saturated filter paper on top of the membrane completed the sandwich. Again air bubbles was removed as before. The second saturated fibre pad was placed on the filter paper. The gel holder was closed and held firmly to keep the gel membrane sandwich from moving laterally and disrupting good contact. The latch was finally secured.

5. The gel holder was placed in the central slot of the transfer tank so that the gray panel of the holder was on the cathode (negative) side of the tank. The cathode is indicated by a black disc on the eletrode panel (located on the inside wall of the tank) and the red disc indicates the anode. The buffer tank was filled to the bottom of the red disc with prechilled transfer buffer. The lid was the put in place.

6. Finally the tank was plugged into the power supply with the red wire (anode) to the red outlet and the black wire (cathode) to the black outlet on the power pack. The transfer was carried on at a constant voltage of 60V for 1 hour, at 30V for overnight and finally at 60V for another 1 hour.
7. After transfer, the cassette was disassembled and the NCM was removed and air-dried. It was used for immunostaining and Indian ink staining.

3.9.3 Procedure:

1. The blotted NCM was washed with PBS – Tween, for 5 mins. The washing was done at room temperature with constant shaking. The washing was repeated 3 times.
2. The NCM was then stained with 1:1000 dilution of Indian ink in PBS-Tween for overnight at room temperature with agitation.
3. Following staining the NCM was rinsed briefly with deionised water. Rinsing for 1 min. or less was sufficient.
4. The NCM was then allowed to dry.

3.10 Immunostaining of NCM blot:

3.10.1 Reagents and buffer:

1. Anti-human Ig antibody-enzyme conjugates (Sigma)

   Anti-human IgG1 antibody-alkaline phosphatase conjugate

   Anti-human IgG2 antibody-alkaline phosphatase conjugate

   Anti-human IgG3, IgG4 antibody-alkaline phosphatase conjugate

2. 1% Bovine serum Albumin (BSA) in PBS
3. 0.1% BSA in PBS
4. 0.05% Tween-20 in PBS (PBS-Tween)
5. 0.1% BSA in 0.05% Tween-20 in PBS (BSA in PBS-Tween)
6. 0.15M Veronal acetate buffer, pH 9.6
7. 5-Bromo-4-Chloro-3-Indolyl phosphate, disodium salt (BCIP)
8. p-Nitro Blue Tetrazolium Chloride (NBT
9. 1.0 M Magnesium chloride ($MgCl_2$)

3.10.2 Procedure:

1. The NCM membrane was air dried and then cut into strips (10.5 x 4mm) and placed in separate wells in multiwell staining tray.
2. 1% BSA in PBS was added to each well and incubated at 37^0C in a shaker for 45 mins.
3. The strips were then washed with 0.1% BSA in PBS for 5 mins.
4. The test serum samples were diluted 1:100 in 0.1% BSA in PBS. The diluted sera was added to the strips and incubated at 37^0C in a shaker for 90 mins.
5. After incubation the strips were washed 3 times with 0.05% PBS-Tween, each wash lasting 10 mins.

6. The conjugate was diluted 1:500 in 0.1% BSA in PBS-Tween. The diluted conjugate was added to the strips and incubated at 37^0C in a shaker for 90 mins.
7. After incubation the conjugate was discarded and the strips were washed with PBS-Tween 3 times, each wash lasting 10 mins.
8. Finally the strips were washed with 0.15M veronal acetate buffer for 5 mins.
9. The buffer was discarded and the substrate was added to the strips. The trays were gently shaken by hand till bands appeared. A positive control serum was used in each test to detect the development of bands after the appearance of bands further reaction was stopped by discarding the substrate and adding tap water. The strips were then air-dried.
10. The developed strips and the marker strips were photographed for analysis.

3.10.3 Analysis

1. The number of bands in each strip was counted.
2. The distances of the bands and dye front from the origin (upper end) were measured and recorded.
3. Rf ndard curve was prepared from the readings of the protein marker strip. Seperate graphs were prepared for each experiment.
4. The molecular masses of the specific bands were measured off the curve.

3.11 PCR:

3.11.1 Extraction of DNA: The blood samples were collected with anticoagulant and buffy coats were separated after – centrifugations. The buffy coats were treated with saponin for the break down of the monocyte. Bone marrow samples of some selected patients before and after treatment were also taken for DNA study. DNA was extracted by using DNA extraction kit (Qagen Tokyo, Japan).

3.11.2 DNA extraction from Bone marrow and Buffy coat: 500 µl of distilled water was added to the 200 µl of Buffy coat then vortexed. 50 µl of saponin and 500 µl of PBS was added and centrifuged after performing vortex. 500 µl of PBS was added for washing and vortexed and centrifuged, 200 µl PBS, 30 µl of proteinase K and 200 µl of AL buffer was added, heated at 70 0C for 10 minutes, 200 µl of ethanol was added. Then transferred into spin column and centrifuged at 8000 g transferred into new column and Buffer AW1 were added and centrifuged at 8000g, transferred into new tube & 500 µl of AW-2 buffer was added and centrifuged. Then, the upper column were transferred in to ependroff and 100 µl of AE buffer was added and centrifugation was done at 8000 g, DNA is being deposited in the ependroff. For DNA extraction from Bone marrow saponin is not needed

3.11.3 DNA extraction from Sand fly: All the procedure followed for DNA extraction from Bone marrow is same for sandfly except AL buffer where instead of AL buffer ATL buffer were added. The primers used are constructed from kDNA of *L. (L) donovani*. DD8 strain to amplify a fragment of 354 bp in length.

3.11.4 Primers used in this study

K upper primer 5' GGG ATT GGA CTT GGT GGA 3'

K lowers primer 5' CAC AGC CCG CAG ATA CAA AT 3'

3.11.5 PCR Reactions: A four hundred and ninety eight-354bp fragment of DNA was amplified. PCR amplification was carried out in a 25µl final volume containing two µl DNA, 2.5 µl 1 x PCR buffer, 1.5 M $Mgcl^2$, 25 µm of each dNTP, 10 pMols of each primer, and 1.25 unit of taq DNA polymarse enzyme. Samples were subjected to initial denaturation at 94^0 C for 10 minutes followed by 35 cycles of 94^0C for 45 sec, 55^0 C for 30 sec and 72^0 C for 1 min and 30 sec. Followed by final extension at 72^0 C for 10 min.

3.11.6 Electrophoresis: A gel was prepared with 1.5-% agarose. After Amplification 10 µl samples of the PCR products and loading buffer was mixed and loaded into each well after electrophoresis the bands were stained with ethidiumbromide and the bands were analyzed and compared to the bands obtained with a positive leishmania DNA control. The bands obtained at 354 bp are identical with the bands, which were shown at 354 bp and sequenced by Shamsuzzamans, *et al.*, (2000).

3.11.7 Statistical analysis: Using the Statistical Package for Social Science (SPSS) program in computer did statistical analysis. Data were presented frequency tables and graphs where applicable. Chi square test and unpaired 't' test was done to find out difference of different variables with related parameter. Multivariate logistic regression analysis was done to find out the predictors of kala azar infection and one-way analysis of variance (within group design) was done to assess the changes of immunolglobin parameters before and after specific treatment of kala azar. A *'p'* value less than 0.05 were considered as significant.

CHAPTER IV

RESULTS

RESULTS

4.1 Introduction:

A total of 449 household were visited and among them 1844 household members were selected for blood sample collection for sero-surveillance of Kala azar. The present study was carried out to investigate the sero-prevalence of *L. donovani* infection and kala-azar (visceral leishmaniasis) in an endemic rural community and to determine the kinetics and characteristics of immune response in pre- and post treatment active kala-azar. The antibody response to *L. donovani* was studied by enzyme linked immunosorbent assay (ELISA) using specific leishmania antigen. It has also been attempted to investigate the role of molecular techniques like PCR as a non-invasive diagnostic tool for leishmania infection. Sandflies of the endemic areas were studied to investigate its involvement in the transmission of diseases. Sandflies were caught, identified and analyzed for detection of *L. donovani* specific DNA by PCR. Therefore, the results of the study have been presented in two sections namely (a) results of sero-epidemiology and (b) results of immunological and molecular studies of kala-azar cases.

4.2 Results of sero-epidemiology:

Ten villages of Fulbaria Upozilla under Mymensing district were included and from each village about 180 respondents were taken by systematic random sampling as described in material and methodology section. Epidemiological

data and blood samples were collected from 1844 people of 449 households of the ten selected villages to determine the sero-prevalence of *L. donovani* infection in rural population of Bangladesh.

4.3 Standardization of Eliza test and determination of diagnostic cut off OD values:

The antibody to *L. donovani* was determined by enzyme linked immunosorbent assay (ELISA) using leishmania specific antigens. Cut off OD values of ELISA test for anti-leishmania IgG antibodies were initially determined to find out the *L. donovani* exposure rate and active cases of kala-azar.

To find out the cut off OD value of ELISA test for anti-leishmania IgG antibodies for determining the sero-positive rate, ELISA was performed with sera from known kala-azar cases, healthy subjects from kala-azar endemic area and from healthy subjects and non kala-azar diseased (TB and typhoid cases) from kala-azar free areas (non-endemic).

Blood was collected from 131 LD bodies/culture positive active kala-azar cases, 48 healthy subjects from kala-azar endemic areas and 150 health subjects from non-endemic areas. The mean OD of the healthy and diseased patients (n=1500 from kala-azar non-endemic area is shown in Table-1.

Diagnostic cut off values for anti-leishmania IgG, IgG1, IgG2, IgG3 and IgG4 were calculated to find out the active cases of kala-azar. Anti-leishmania IgG, IgG1-IgG4 antibodies were determined by ELISA using specific antigen (K-

39). The mean OD of the sera of healthy subjects from kala-azar endemic areas +3x SD of the tests was taken as cut off OD to determine the sero-positive cases or active kala-azar case. The respective cut off OD values for IgG and IgG sub-classes are shown in Table-2.Any test sample giving OD above these calculated cut off values was considered positive and indicated either sero-positive or active disease and referred to as exposed to *L.donavani* infection.

The sensitivity and specificity of ELISA test for the anti-leishmania IgG and IgG1 antibodies were 97% and 100% respectively having a cut off value of 0.87 (Table-2).

Only total anti-leishmania IgG was used to determine the sero-positivity and active kala-azar cases as all sub-class specific anti-leishmania antibodies were not tested in all 1844 samples.

Table-1: Mean OD values of total anti-leishmania IgG antibodies by ELISA of healthy subjects from non-endemic areas

Anti-leishmania antibody	Types of Subjects*	Total No	Mean OD±SD
Total IgG	Healthy	100	0.10±0.09
	TB cases	25	0.10±0.06
	Typhoid	25	0.07±0.03
	Total	150	0.09±0.08

* Subjects were from kala-azar non-endemic area.

Table-2: Diagnostic cut off value of ELISA test for total anti-leishmania IgG and IgG subclasses and Sensitivity and specificity of ELISA test for detection of anti-leishmania IgG and IgG1 antibodies.

*Healthy people from endemic area

Anti-leishmania antibodies	Total study population* (N)	Mean OD±SD	Cut off OD value (Mean+3xSD)
Total IgG	1539	0.50±0.13	0.50+3x0.13=0.87
IgG1	48	0.06±. 09	0.60+3x0.09=0.87
IgG2	48	0.52±0.11	0.52+3x0.11=0.84
IgG3	48	0.47±0.14	0.47+3x0.14=0.89
IgG4	48	0.52±0.15	0.52+3x0.15=0.97

Test	Cut off OD	Sensitivity (%)	Specificity (%)
ELISA for IgG	0.87	97.7	100
ELISA for IgG1 Antigen- K39	0.88	97.7	100

Note: Sensitivity= True positive/True positive + False negative; Specificity= True negative/False positive + True negative

4.4 Sero-prevalence of anti-leishmania IgG antibodies and its relation with clinical status:

The cut off value as calculated above was then used to determine the sero-positivity of the study population and was correlated with clinical status of the subjects. Detail clinical history was taken from all subjects having OD value greater than the cut off points (0.87).

Based on the cut off value, it was observed that out of 1844 household members, 114(6.2%) individuals were sero-positive or exposed to *L.* Out of these 114, the clinical status of 70 had active kala-azar disease as determined by the clinical features and confirmed by further laboratory investigation (demonstration of parasites). Therefore, based on the level of anti-leishmania antibodies and diagnostic cut off value, 3.8% (70/1844) people were found suffering from active kala-azar in the study area (Table-3). So, the remaining 44 individuals were then considered as exposed to *L.*dinavani infection only without clinical kala azar at the time of study.

Figure-1 shows the mean IgG OD values of active kala-azar cases and sero-positive or exposed cases compared to sero-negative individuals (1730) and non-endemic controls. The mean OD values of active kala-azar cases and leishmania-exposed cases were significantly high ($p<0.05$) compared to sero-negative healthy individuals and controls.

Table-3: Sero-prevalence of anti-leishmania IgG antibodies and its relation with clinical status

Total Population	Total Sero-positive (OD >0.87)	Total Sero-negative (OD<0.87)	Clinical status of sero-positivity cases	Total number
	N (%)	N (%)		No
1844	114 (6.2%)	1730 (93.8)	Healthy without disease Active KA	44 70
			Total	114

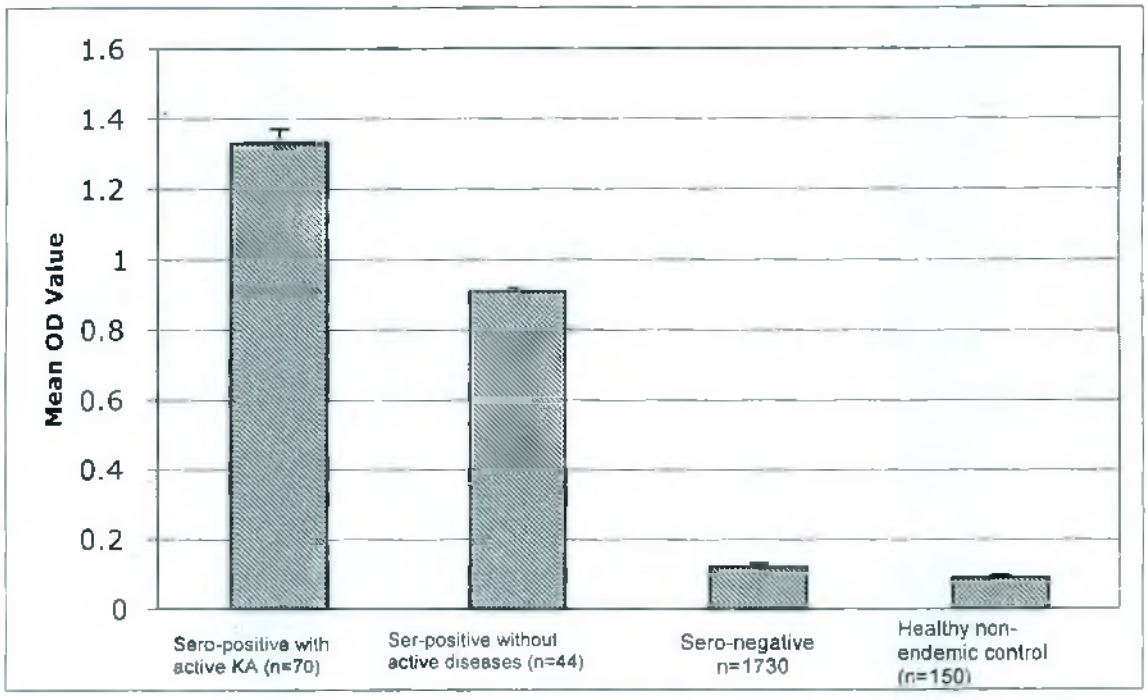

Figure-1: Mean OD values of anti-leishmania IgG antibodies of kala-azar and exposed cases compared to healthy endemic and non-endemic controls.

Table-4 shows the detail distribution of leishmania sero-positive and active kala-azar cases in study villages of five unions of Fulbaria upazilla. All the five Unions of the Fulbaria Upozilla were affected. Fulbaria was the most affected Union (11.25%) while Balian Union was least affected (8.33%).

Table-4: Distribution of sero-positve and active kala-azar cases in 5 Unions of study area.

Name of the Union With population	Total study population (N)	Total No. Sero-positive		No sero-positive	
				Without active KA	With active KA
		No.	(%)	N	N
Fulbaria – 48065	382	43	11.25	25	18
Kushmail – 37248	382	42	10.99	27	15
Bakta – 33497	360	38	10.56	25	13
Putijana – 33717	360	36	10.00	23	13
Balian – 32268	360	30	8.33	19	11
Total	**1844**	**189**	**10.2**	**119**	**70**

Note: - KA: Kala-azar

4.5 Distribution of sero-positive cases by family:

Figure-1 shows the mean OD values of active kala-azar cases and sero-positivity or exposed cases compared to healthy endemic and non-endemic controls. The mean OD values of active kala-azar cases and leishmania-exposed cases were significantly high ($p<0.05$) compared to controls.

Among the infected families, mean number of infected member was 1.4±0.7 (rang 1 to 5). Of the 134 households affected, 70.3% households had one infected member while 19.7% and 9.8% houses had two and three or more members infected respectively (Table-5).

Table-5: Distribution of sero-positivite cases per affected households

No. of members infected per households	Household (N=81)	
	No	%
One	57	70.3
Two	16	19.7
More than three	8	9.8%

4.6 Estimated sero positive and active kala azar cases in study area and entire Fulbaria Upazila:

Based on the sero-positivity rate and diagnostic cut off value, the burden of the *L. donovani* infection has been estimated in the study area. An estimated 18.849 people were exposed to *L. donovani* infection in five unions of study

area while estimated active kala azar cases were 7022 (Table-6). Therefore, an estimated 43,757 and 12,869 people had exposure to *L. donovani* infection and active kala-azar respectively in entire Fulbaria Upazila of Mymensing district having a population of 428,998.

Table-6: Estimated kala azar cases in 5 Unions of study area

Name	Total Population	Estimated exposed cases	Estimated Kala-active azar cases
Fulbaria	48065	4903	1826
Kushmail	37248	3799	1415
Bakta	33497	3417	1272
Putijana	33717	3439	1281
Balian	32268	3291	1226
Total (5 Unions)	**184795**	**18847**	**7022**

Note: There are eleven Unions in Fulbaria Upazila having a total population of 428,998. So the estimated estimated exposed and active kala-azar cases in the entire Fulbaria upzazila was 43,757 and 12,869 respectively

4.7 Characteristics of Kala azar positive cases, Socio-economic and demographic information:

Analysis of age distribution of *L. donovani* sero-positive cases indicates that all age groups were almost equally affected. But, age group 20-29 was most affected (13.0%) while people between 10-19 and over 60 years of age were

least exposed to infection (7.8% and 7.7%). However, the difference was not statistically significant ($p>0.05$). No significant association of *L. donovani* infection and sex had been observed ($p >0.05$). The detail of age and sex distribution of the study population is given in Table-7 and Table-8 respectively.

No statistically significant association was found with the level of education with that of exposure to leishmania infection indicating that all types of people were equally exposed (Table-9).

The occupational status of the study population has been shown in Table-10. No significant association was observed with occupation and sero-positive cases. The infection rate ranged between 7.7-19.8% among different occupational groups (Table-10). However, sero-positive cases were higher among dependent members of the family (19.8%) followed by unemployed members (16.7%) and farmers (12.8%).

It was seen that out of 449 families, majority (81%) had income less than Tk. 4000 per month (Table-11). Also, Table-11 shows that all income groups were equally exposed to leishmania infection.

Table-7: Age distribution of *L. donovani* exposed sero-positive cases

Age	Total Study population (N)	Sero-positive for L. donovani	
		Number	%
<10	371	41	11.1
10-19	447	35	7.8
20-29	292	38	13.0
30-39	329	37	11.2
40-49	207	21	10.1
50-59	94	9	9.6
≥60	104	8	7.7
Total	**1844**	**189**	**10.2**

Table-8: Sex distribution of *L. donovani* exposed sero-positive cases

Sex	Total Study population (N)	Sero-positive for L. donovani	
		Number	%
Male	865	97	11.2
Female	979	92	9.4
Total	**1844**	**189**	**10.2**

Note: p>05

Table 9: Educational status of study population and *L.donovani* exposed sero-positive cases

Level of education	Total Study population (N)	Sero-positive for L. donovani	
		Number	%
No schooling	628	72	11.5
Non formal education	230	22	9.6
Grade I-V	509	55	10.8
Grade VI-X	331	27	8.2
SSC	67	4	6.0
HSC	45	8	15.6
Graduate and above	16	1	6.3
Total	**1826**	**189**	**10.2**

Note: p>05 by χ^2 test

Table-10: Occupation of study population and *L.donovani* exposed sero-positive cases

Occupation	Total Study population (N)	Sero-positive for L. donovani	
		Number	%
Unemployed	30	5	16.7
Farmer	352	45	12.8
Service holder	16	2	12.5
Housewife	546	56	10.3
Day laborer	39	3	7.7
Business	36	4	11.1
Student	628	55	8.8
Dependent	86	17	19.8
Others	13	2	15.4
Total	**1846**	**189**	**10.8**

Note: p>05 by χ^2 test

Table-11: Family income of study population and *L. donovani* sero-positive cases

Family income per month	Number of family (%)	Sero-positive for L. donovani (%)
≤2000	181 (40.3)	25.4
2001-4000	188 (41.8)	32.6
4001-6000	42 (9.3)	35.7
≥6001	38 (8.4)	23.7

Note: p>05 by χ^2 test; *n=449*

4.8 Household information:

Analysis of housing conditions and the surrounding environment of the study population revealed that most of the of houses were mud-houses (80%) and followed by tin-houses (11.0%). No statistically significant association was found in terms of housing condition and sero-positivity of the household members (Table-12). The anti-leishmania IgG sero-positive cases were found to be high among the houses having cracks in the wall or floor (36.0%) compared to houses without crakes (Table 13). Exposure to leishmania parasite was almost equal (31.4% vs. 27.5%) among the occupants of houses with or without cow shed around homesteads (p>0.05; Table 14).

Table-12: Housing condition of study population and sero-positive families

Housing condition	Number of houses (N=449)	% Of houses with sero-positive cases
Roof		
Thatched/bamboo	94	33.0
Tin	355	29.0
Wall		
Thatched/bamboo	27	22.2
Mud	360	30.3
Tin	52	32.7
Brick	10	20.0
Floor		
Mud	443	30.0
Brick	6	83.3

Note: p>05 by χ^2 test

Table-13: Relation between presence of cracks in walls and floors of houses and exposure to *L. donovani* infection

Cracks	Total households (N=449)	% Of houses with sero-positive cases	P value of chi-square
Present	178	36.0	0.022
Absent	271	25.8	

Table 14: Location of cow shed adjacent to households and exposureto *L. donovani* infection

Cow shed near households	Total households (N=449)	% of houses with sero-positive cases	p value of chi square
Present	271	31.4	0.385
Absent	178	27.5	

In the present study, an attempt was also made to assess the awareness of the family regarding the disease "kala-azar". Table-15 shows that 87.8% of the respondents were aware of the disease "kala-azar". The main sources of information were health workers (44.2%) followed by physicians (21.3%), radio (20.6%), television (20.6%), neighbors (12.7%), relatives, etc. It was interesting that only 1% had the knowledge that the disease is spread by sand fly. Majority had the notion that it was spread by contaminated food (Table-15).

Table-15: Knowledge of study population regarding kala-azar disease

Variables	Number	Percent
Ever heard kala azar		
Yes	394	87.8
No	55	12.2
Total	449	100.0
****Sources of information***		
Health worker	174	44.2
Doctors	84	21.3
Radio	81	20.6
TV	74	18.8
Neighbors	50	12.7
Relatives	22	5.6
News Paper	2	0.5
The diseases is acquired by		
Sand fly bites	48	1.0
Mosquito bites	300	66.8
Food	101	22.5

***Multiple responses**

Out of 449 households surveyed by serology, one or more members of the 18.0% (81/449) of households were found positive for *L. donovani* antibodies or exposed to the parasite (Figure-2).

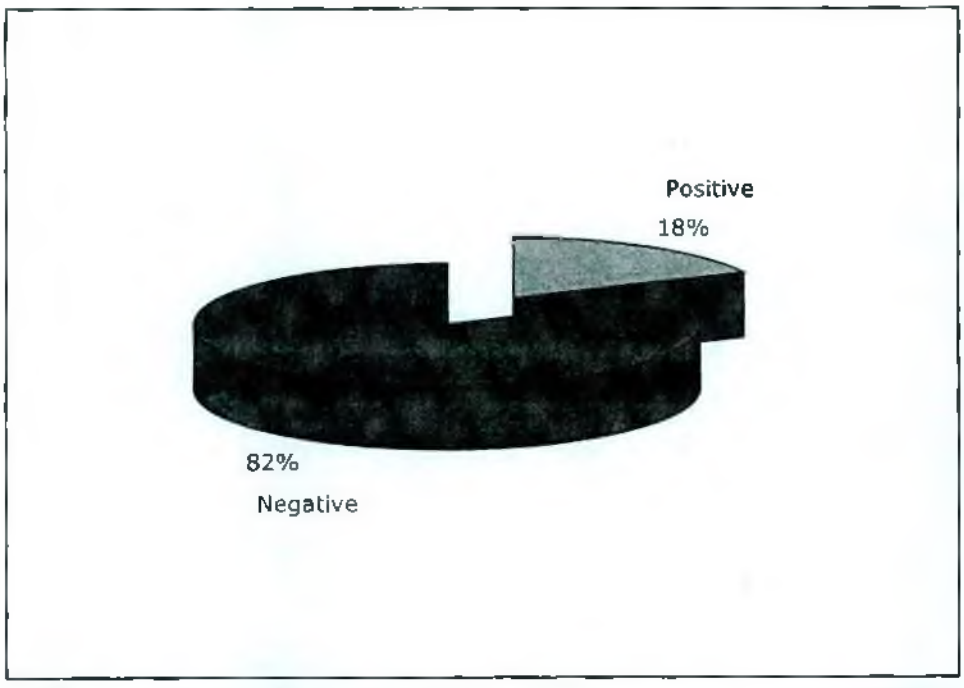

Figure 2: Families (Households) positive for *L. donovani* antibodies

4.9 Microscopic, bone marrow and immunological results of kala azar

Results of immunogical and molecular studies of kala-azar cases.

Characteristics of Kala azar positive cases

A total of 131 kala-azar cases were included in the study. Seventy active kala azar positive cases from the sero-surveillance and another 61 cases were included from others areas of kala-azar endemic Mymensingh and Gazipur districts. All the Ld body/culture positive cases were given standard treatment as recommended by WHO.

Age and sex distribution of the 131 KA patients has been presented in Table 16. The majority of the population (33.6%) was between 10-19 years of age.

The mean age of the subjects was 23.3±13.5 years. Sex distribution of the patients showed that the majority of the patients were male (71.8%) while only 28.2% were female.

Table 16: Age and sex distribution of the 131 kala azar patients

Age in years	*Total Study population (N)*	*Number of Study subjects*			
		Male		*Female*	
		N	*%*	*N*	*%*
<10	13	9	69	4	31
10-19	44	32	73	12	27
20-29	33	20	61	13	39
30-39	25	20	80	5	20
40-49	13	8	89	1	11
>50	7	5	71	2	29
Total	**131**	**94**	**71.8**	**37**	**28.2**

4.10 Microscopic, bone marrow and immunological results of kala azar positive cases

The results of the microscopy and culture of the bone marrow and separated buffy coat specimens have been given in Table 17. It has been found that all 131 clinically suspected cases of kala-azar were positive for LD bodies by microscopy in the bone marrow smears. But the culture of bone marrow was positive in 74% cases. All the buffy coat samples were negative for parasites both by microscopy and culture in NNN media. The result of the IgG and its subclasses assay of 131 kala azar patients have been shown in Table 18 where it has been shown that total IgG and IgG1 antibody levels were significantly

pronounced. The IgG2, IgG3and IgG4 anti K-39 antibody responses were same as endemic healthy control subjects.

Table 19 shows the diagnostic value of IgG and IgG1 antibodies in relation to microscopy and culture of bone marrow of 131 kala-azar cases. Three cases were negative in both IgG and IgG1 ELISA tests. Since the mean OD values of anti-leishmania IgG2, IgG3 and IgG4 using K-39 antigen were not significantly different from the OD values of healthy control subjects of endemic area, these antibody markers could not be used for diagnosis of active kala-azar cases.

Table 17: Results of direct microscopy, culture of bone marrow and peripheral blood buffy coat of 131 clinically suspected kala-azar cases

Results	Culture of		LD body by microscopy N (%)
	Bone Marrow N (%)	*Buffy Coat* N (%)	
Positive	96 (74)	00	131 (100)
Negative	35 (26)	131(100)	0
Total	131	131	131

Note: LD body = Leishmania donovani body

Table 18: Anti-leishmania IgG and IgG subclass antibodies in Kala-azar patients and control subjects at the time diagnosis.

Anti-leishmania antibodies	Study subjects	Mean OD ± SD	p value
IgG-Total			
Kala-azar patients	131	1.56 ± 0.31	0.001
Non-endemic healthy controls	150	0.09±0.07	
Endemic healthy controls	48	0.50±0.13	
IgG-1			
Kala-azar patients	131	1.96±0.48	0.001
Non-endemic healthy controls	150	0.05±0.02	
Endemic healthy controls	48	0.0.60±0.0.09	
IgG-2			
Kala-azar patients	131	0.62±0.22	0.001
Non-endemic healthy controls	150	0.05±0.02	
Endemic healthy controls	48	0.52±0.11	
IgG-3			
Kala-azar patients	131	0.46±0.26	0.001
Non-endemic healthy controls	150	0.05±0.02	
Endemic healthy controls	48	0.47±0.14	
IgG-4			
Kala-azar patients	131	0.19±0.21	0.001
Non-endemic healthy controls	150	0.5±0.01	
Endemic healthy controls	48	0.51±0.14	

Table 19: Anti-leishmania IgG and IgG1 serology in relation to culture & direct microscopy

Test	Total No	Positive for anti-leishmania IgG by ELISA No	Positive for anti-leishmania IgG1 by ELISA No
Positive for LD bodies by microscopy (n=131)	131	128	128
Culture Positive (n=96)	96	95	95
Culture Negative (n=35)	35	33	33

Note: Three cases had OD value for IgG and IgG1 below the cut off value of 0.87. K-39 antigen was used in ELISA

4.11 Immunological Follow Up: Post-treatment kinetics of anti-leishmania antibodies:

Serum samples were collected from kala-azar patients following treatment. Sera were collected after 15 days, 3, 6, 9 and 24 months after treatment. The IgG and IgG1 antibody levels before and post treatment at various time interval have been shown in Figure-3 and Figure-4. The IgG and IgG1 declined in the serum of the patient after treatment with the passage of time. The kinetics of the IgG2, IgG3 and IgG4 antibody levels in relation to treatment are shown in Figure-5, 6 and 7. It is apparent that there was no increase of the IgG2, IgG3 and IgG4 in *L. donovani* infected active kala-azar patients compared to healthy individuals. The level remained at the background level prior to and following treatment through out the study period.

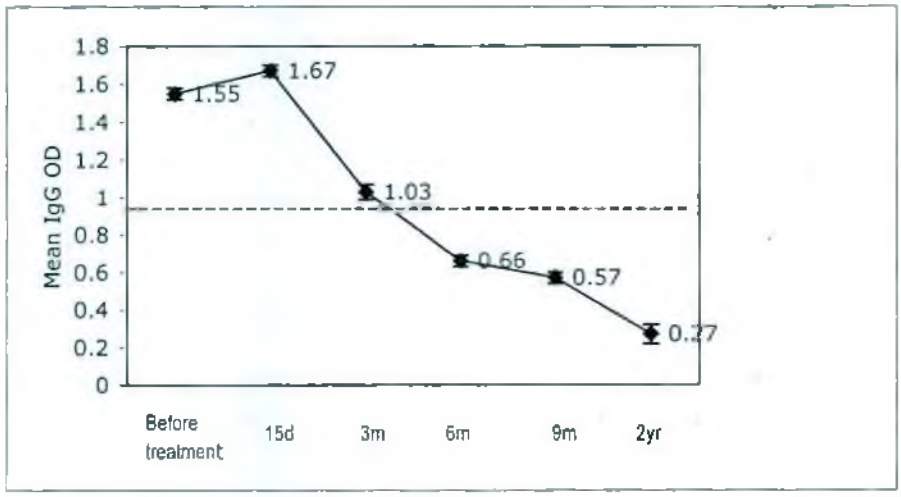

Figure-3: Anti-leishmania IgG antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21). The dashed line indicates the diagnostic cut off OD value (0.87). $p< 0.05$ for before treatment vs 3 and 6 months.

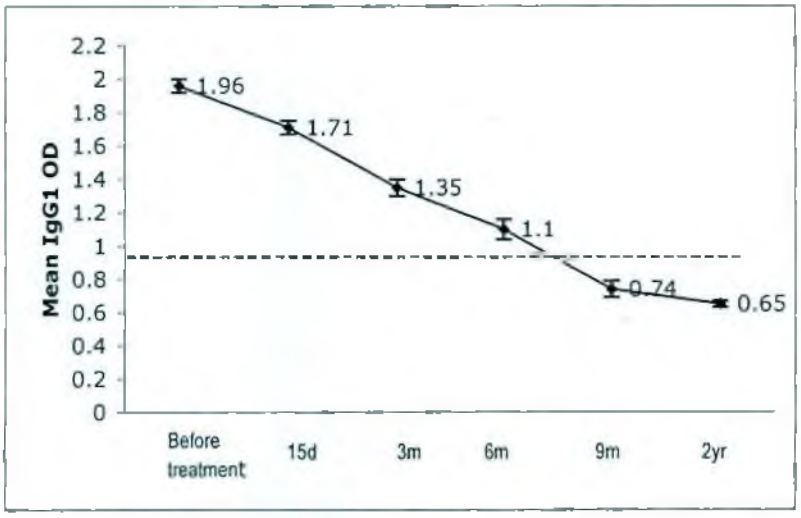

Figure-4: Anti-leishmania IgG1 antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21). The dashed line indicates the diagnostic cut off OD value (0.89). $p < 0.05$ for before treatment vs 3 and 6 months

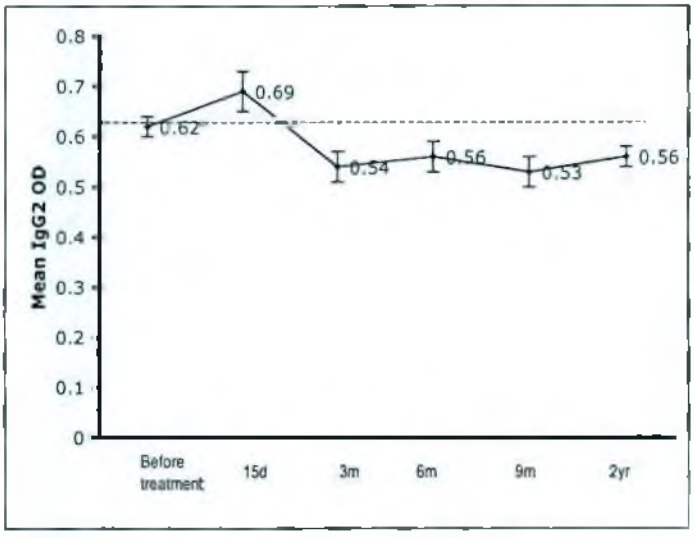

Figure-5: Anti-leishmania IgG2 antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21). The dashed line indicates the diagnostic cut off OD value (Mean OD of endemic healthy subjects + 1SD; 0.62). $p > 0.05$ for before treatment vs 3 and 6 months

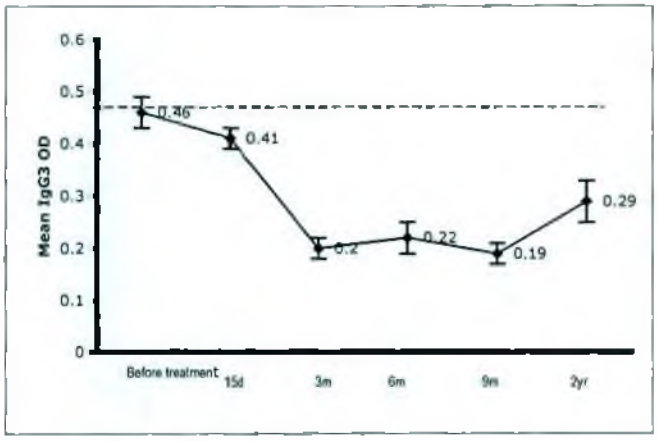

Figure-6: Anti-leishmania IgG3antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21). The dashed line indicates Mean OD of endemic healthy subjects 0.47). P<0.05 for before treatment vs. 3 and 6 months

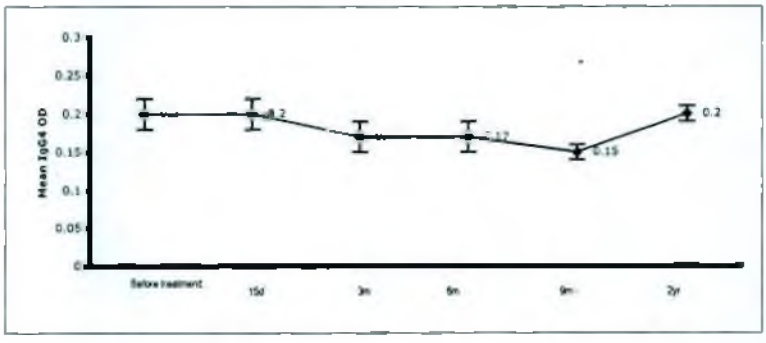

Figure-7: Anti-leishmania IgG4antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21). P>0.05 for before treatment vs. 3 and 6 months.

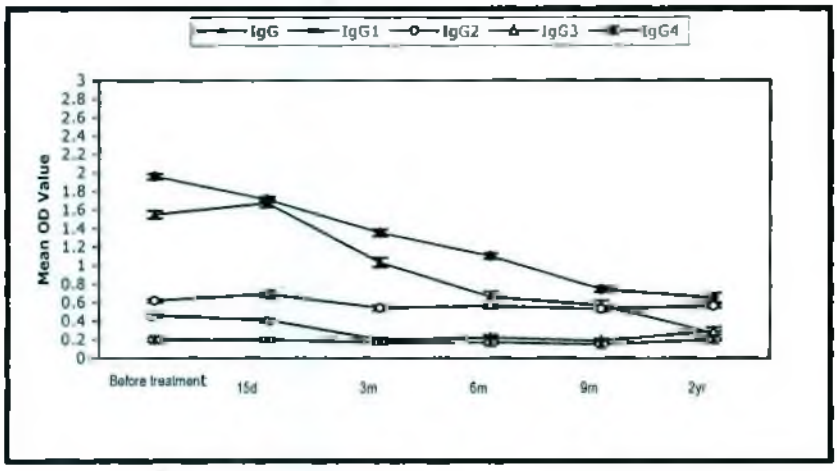

Figure-8: Anti-leishmania IgG antibody level in kala-azar patients before treatment (n=131) and after treatment at 15days (n=121), 3months (n=121), 6 months (n=106) 9 months (n=78) and 2 years (n=21).

Table-20 shows the cumulative conversion of anti- leishmania IgG and IgG1 serology test over 9 months period. Following treatment. A total of 77 kala-azar cases were followed over 9 months period and anti-leishmanai IgG and IgG1 were determined at 3, 6 and 9 months following treatment. It is apparent from the Table-20 that 94% patients became IgG negative (titre below cut off OD of 0.87) by 9 months while for IgG1 it was 80%. All remaining positive cases became negative by 2 years following treatment.

Table-20: Sero-conversion rate for anti-leishmania IgG and IgG1 antibodies following treatment

Test	% Negative after (cumulative)			Remained positive after 9 months
	3month N (%)	6 months N (%)	9 months N (%)	N (%)
IgG (n=77)	40 (51.9)	60 (77.9)	73 (94.8)	4 (5.2)
IgG1 (n=77)	22 (28.6)	31 (40.3)	61 ((79.2)	16 (20.8)

4.12 Anti-leishmania antibodies against soluble antigens

Anti-leishmania IgG1-IgG4 antibodies were determiuned using soluble antogens by ELISA. Similar to the K-39 antigens, no antibody response was noted for IgG2-IgG4. Only there was increase of IgG1 antibody in patients. But the titer was much less compared to K-39 antigen. The detail is given in Table-21.

Table-21: Total anti *L. Donovani* serology by soluble antigen in relation to kala azar patient and endemic control

Test	Total No	Positive No %	Serology-IgG-1 Mean OD
IgG1			
KA patient	48	15	0.30±0.25
Endemic control	24	01	0.12±0.08
IgG2			
KA patient	48	48	0.25±0.19
Endemic control	24	24	0.36±0.25
IgG3			
KA patient	48	48	0.15±0.11
Endemic control	24	24	0.06±0.02
IgG4			
KA patient	48	48	0.15±0.09
Endemic control	24	24	0.06±0.04

Cut off=0.12+0.24=0.36 (Mean+3xSD) in 1844 persons 70 positive=3.79%

ELISA using K-39 antigen was used to test sera from 35 kala-azar positive cases for the presence of anti-leishmania IgE antibodies. Table 22 shows that there was no significant increase or decrease of antigen specific IgE level in patients having active kala-azar and healthy control subjects. Also, the IgE level was same in patients before and after treatment (Table-22)

Table 22: Total anti-leishmania IgE antibodies by K-39 antigen in kala-azar patients and in endemic healthy control subjects

Test	Total No	Serology-IgE-T Mean OD
KA patient before treatment	35	0.07±0.03
KA patient after treatment	33	0.06±. 018
Endemic control	22	0.06±0.02

Note: p>0.05; compared between kala-azar patients and control; p>0.05 compared between before and after treatment.

The ELISA using K-39 antigen was compared with commercial immunochromatography test for anti-leishmania antibody test kit. A total of 131 ELISA positve samples were testted. All were also found positive by ICT method. Table-23 shows that comparison of ELISA & ICT.

Table 23: Result of comparison between Elisa & ICT

No ELISA postive*	Positive by ICT N (%)	Negative N (%)
131	131 (100)	0

* ELISA with K-39 antigen

4.13 Factors affecting the kala azar infection: Multivariate analysis:

In the present study, an attempt was made to assess the socio-cultural factors that might influence the kala azar infection among the population. Current age, sex, marital status, religion, occupation, level of education, housing condition and use mosquito net of the study population were entered into multivariate logistic regression model. Analysis revealed that male sex, mud house and no or irregular use of mosquito net appeared to be important predictors of kala azar infection ($p<0.05$). The kala zar infection was 2.5 times higher among the male, 2.8 times in people living in mud house and 9.1 times higher among the people who had habit of no or irregular use of mosquito net. Current age, marital status, religion, occupation and level of education of the respondents did not appear any significant on kala azar infection ($p>0.05$).

Table-24: Factors affecting the Kala azar infection: Multivariate analysis

Independent variables	*β*	*p value*	*Odds ratio*	*95% CI*
Age in years				
<25 (RC)	-	-	-	-
≥25	0.0927	0.8217	1.0971	0.4899-2.4569
Sex				
Female (RC)	-	-	-	-
Male	0.9278	0.0308	2.5290	1.0895-5.8706
Marital status				
Unmarried (RC)	-	-	-	-
Married	-0.7419	0.1217	0.4762	0.1861-1.2186
Religion				
Hinduism RC)	-	-	-	-
Islam	0.4192	0.5070	1.5208	0.4408-5.2472
Occupation				
Housewife(RC)	-	-	-	-
Manual job	-0.1137	0.8447	0.8925	0.2860-2.7849
Non manual job	-0.7930	0.1806	0.4525	0.1417-1.4445
Level of education				
Illiterate	-0.8303	0.1489	0.4359	0.1412-1.3458
Primary	0.2898	0.5740	1.3362	0.4864-3.6707
Secondary and above (RC)	-	-	-	-
Housing condition				
Non-mud house (RC)	-	-	-	-
Mud house	1.0260	0.0043	2.7899	1.3801-5.6396
Use of mosquito net				
Regular use (RC)	-	-	-	-
No or irregular use	2.2149	0.0000	9.1609	4.6796-7.9336
Model chi square	188.913			
Df	10			
Significance	0.0000			
N	341			
Constant	-3.5925			

4.14 Repeated measure analysis of IgG level in different visits:

The selected patients were followed for certain period to assess their IgG level after specific treatment for Kala azar. The mean IgG-T level before treatment was 1.45 OD and after treatment it was increased to 1.82 (15^{th} day of visit) then decreased to 1.07 (3^{rd} month), 0.68 (6^{th} month) and 0.57 (9^{th} month). The ANOVA shows that these mean OD of IgG and its subclasses are significantly different, $F(4, 240) = 133.019$; $p<0.001$. Repeated measures ANOVA shows that level 1 vs. level 2, level 2 vs. level 3 and level 3 vs. level 4 and level 4 vs. level 5 were found to be statistically significant ($p<0.001$) (Table 27). It was evident that only 21 patients were followed up to 2 years that they had good compliance of treatment. Repeated measure one way analysis of variance (within subjects) reveled that the mean IgG-T level before treatment was 1.45 and after treatment it was increased to 1.82 (15^{th} day of visit) then decreased to 1.07 (3^{rd} month), 0.68 (6^{th} month), 0.57 (9^{th} month) and 0.27 (at 2 year). The ANOVA shows that these mean OD of IgG and its subclasses are significantly different, $F(5, 100) = 68.575$; $p<0.001$. In Table-27 Repeated measures ANOVA shows that level 1 vs. level 2, level 2 vs. level 3 and level 3 vs. level 4 were found to be statistically significant ($p<0.001$), but level 4 vs. level 5 was not statistically significant ($p>0.05$), further it was found to be significant at level 5 vs. level 6 ($p<0.001$).

Table-25: One way repeated measure ANOVA of IgG-T level in different follow up visit (n=61)

a) Descriptive statistics of the IgG T

IgG T level	Mean ± SE	95% confidence interval
Before treatment (Level 1)	1.42±0.05	1.311-1.524
15th day (Level 2)	1.70±0.04	1.614-1.778
3rd month (Level 3)	0.94±0.05	0.837-1.041
6th month (Level 4)	0.73±0.04	0.655-0.807
9th month (Level 5)	0.60±0.04	0.526-0.681

b) Tests of Within-Subjects Contrasts

FACTOR	Type III Sum of Squares	df	Mean Square	F	Sig.
Level 1 vs. Level 2	4.732	1	4.732	20.856	0.000
Level 2 vs. Level 3	34.933	1	34.933	163.703	0.000
Level 3 vs. Level 4	2.646	1	2.646	22.172	0.000
Level 4 vs. Level 5	0.984	1	0.984	10.481	0.002

***p<0.001

Table-26: One way repeated measure ANOVA of IgG-T level in different follow up visit (n=21)

a) Descriptive statistics of the IgG T

IgG T level	Mean ± SE	95% confidence interval
Before treatment (Level 1)	1.45±0.10	1.233-1.679
15th day (Level 2)	1.82±0.5	1.717-1.907
3rd month (Level 3)	1.07±0.10	0.863-1.286
6th month (Level 4)	0.68±0.07	0.531-0.832
9th month (Level 5)	0.57±0.06	0.449-0.701
2 year (Level 6)	0.27±0.05	0.164-0.396

b) Tests of Within-Subjects Contrasts

FACTOR	Type III Sum of Squares	df	Mean Square	F	Sig.
Level 1 vs. Level 2	2.668	1	2.668	11.240	0.003**
Level 2 vs. Level 3	11.426	1	11.426	39.608	0.000***
Level 3 vs. Level 4	3.253	1	3.253	23.450	0.000***
Level 4 vs. Level 5	0.235	1	0.235	3.371	0.081
Level 5 vs. Level 6	1.844	1	1.844	18.266	0.000***

****p<0.01; ***p<0.001**

4.15 PCR Results:

From the 131 study samples, 35 cases were selected randomly for PCR and DNA study. Of the 35 samples studied 20, (twenty) were amastigote positive in the Giemsa stained smears but they were culture negative. Other 15 (fifteen) samples were culture positive. All the control samples were negative for ICT and ELISA. Blood samples of these 35 cases were processed in the laboratory and buffy coat were separated and inoculated into culture media they were negative for leishmania promastigote. Of the 35 buffy coat samples 33 were positive by PCR by the primer set used (Table-27). All the samples were antibody positive. Thirty-three (33) samples were positive in PCR showed bands of DNA at 354 bp by the primers constructed from kinetoplast. Of the nuclear DNS sequence of identical bp bands were done by Samsuzzaman et al 2001. Buffy coat from 8 respondents after 15 days of treatment, were also studied. All of them were negative by PCR. (Table-28) Bone marrow of the 5 patients preserved in normal saline was studied from the pre-treated patients.

All these 5 patients showed specific bands at 354 bp. Promastigote from the culture were also extracted and PCR method was done which showed the same identical band and used as positive control.

Table-27: Result of Culture and PCR of Bone-marrow and Buffy-coat

Examination	Culture +ve	PCR +ve
Bone marrow (n= 5)	5	5(100%)
Buffy coat (before treatment)- n=33	0	33(100%)
Buffy coat (post-treatment)- n=15	0	0

One after 15 days, 5 after 3 months & 2 after 6 months of treatment

Table-28: Result of Culture & PCR

Specimens	Culture +ve	PCR +ve
Buffy coat (before treatment, n=30)	0	30(100%)
Buffy coat (post-treatment, n=6)*	0	0
Bone marrow (n= 4)	4	4(100%)

4.16 Direct Microscopy and Culture and PCR results:

A total of 131 patients suspected of having kala-azar were recruited for molecular study. Kala-azar was diagnosed either by demonstrating LD bodies in bone marrow or by its positive culture. All 131 clinically suspected cases of kala-azar were positive for LD bodies in the bone marrow specimens. But culture was positive in 74% cases. Out 131 cases, we have selected 30 cases for molecular diagnosis by PCR. Of the 30 samples studied, twenty were amastigote positive by microscopy but they were culture negative. Other 10

samples were culture positive. From the blood samples of these 30 cases, buffy coats were separated and inoculated into culture media. All 30 were negative in culture. In Table-28 of the 30 buffy coat samples, 30 were positive by PCR which showed bands of DNA of 354 bp. PCR was also done with buffy coat from 6 treated cases. All of them were negative by PCR. Bone marrow of 4 culture positive cases was also subjected to PCR. All were found positive. In Table-29 result of OD value in culture & microscopy positive cases were shown. .

In Table-30 result of clinical features of 189 ELISA positive cases were presented.

Table-29: Result of OD value in culture & microscopy positive case

	Total Case	Positive Case	Finding	OD value
Microscopy+ve	131	131	100%	0.46
Culture+ve	131	96	74%	0.46
Microscopy-ve	131	00	0%	0.46
Culture-ve	131	35	26%	0.46

Table-30: Result of clinical features of 189 ELISA positive cases.

	Total case	Positive case	Finding
Fever	189	10	5%
Spleen	189	80	42%
Liver	189	05	2%
Lymphnode	189	00	00%
Anaemia	189	90	47%
Skin changes	189	85	44%

4.17 Sand fly examination: An attempted was made to extract DNA from the sand fly, which were caught from the houses of kala-azar affected patients. In one extraction 10 sand flies were used. After doing PCR similar bands were detected from the DNA extracted from this sand fly. Which indicates some of the sand fly in this extraction procedure were positive for promastigote. Several workers also tired before to find promastigote in sand fly by other techniame. But we have extracted DNA from the sand fly and subsequent PCR showed this sand fly were having promastigote. Table 31, shows the pattern of sand fly caught from the dwelling of kala azar patient

Table-31: Types of sand fly found in the dwelling of kala azar patient (n=68)

Name	Male	Female	Total	%
P. argentipes	8	21	29	43
S. babu	6	26	32	47
S. sorti	1	6	07	10
Total	**15**	**53**	**68**	**100**

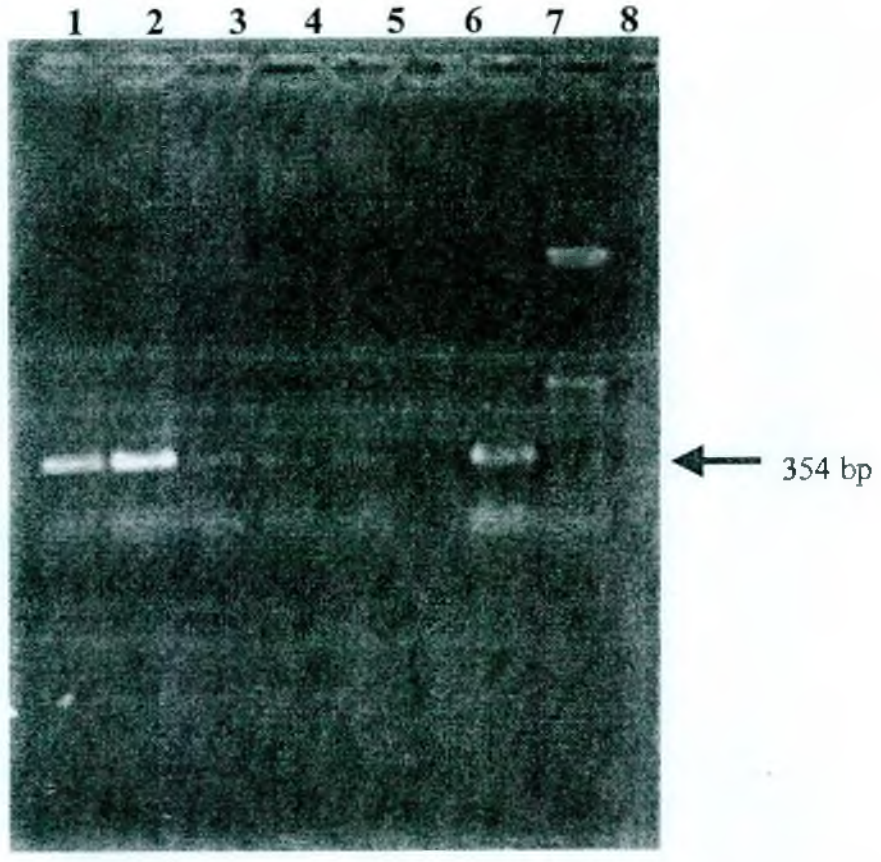

Fig –4: 354 bp product, 1 –Sand fly, 2 –Sand fly, 3 –Buffy coat, 4 –Buffy coat, 5 –Bone marrow, 6 –Negative control, 7 –Promastigote (Positive control), 8 – Ladder.

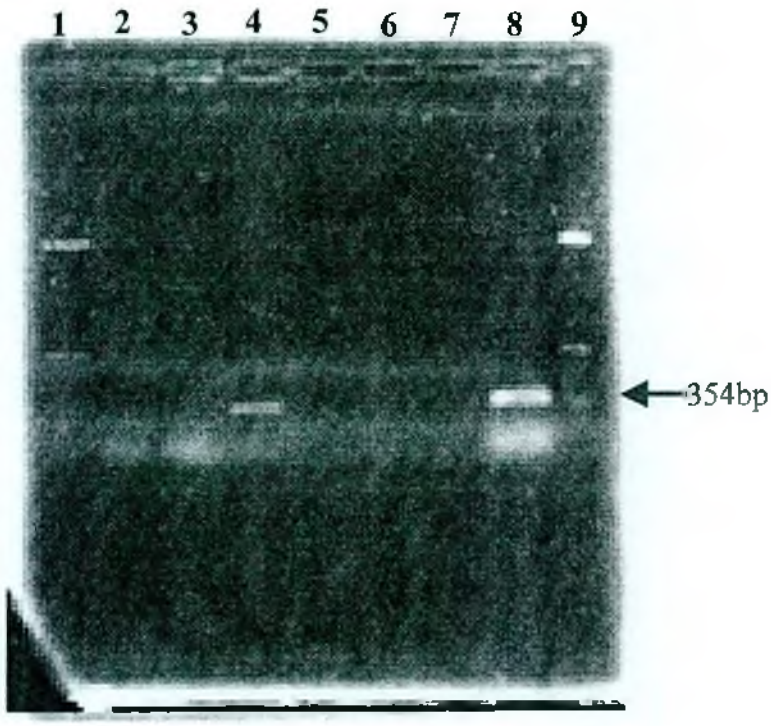

Fig -5: 1 –Ladder, 2, 3, 5 –Three months after treatment, 4 –Before treatment, 6 – Three months after treatment, 7 –Negative control, 8 –Promastigote (Positive control), 9 –Ladder.

4.17 Western Blot:

Immunoblot reactive of Kala azar positive sera obtained from Kala–azar patients showed variable pattern of reactivity. Although the majority of the serum recognized certain immune bands around the 60 to 63 KDa and 28-KDa region. The IgG-3 antibodies showed broad reactivity to leishmanial antigen predominantly in the region between 14 and 34 KDa while the IgG-4 isotype Failed to show any significant reactivity to leishmanial antigen. In reactive phase, IgG-1 showed reactivity particularly at 60 to 63 KDa and 20 to 22 KDa and same with the IgG-2.

failed to show any significant reactivity to leishmanial antigen. In reactive phase, IgG-1 showed reactivity particularly at 60 to 63 KDa and 20 to 22 KDa and same with the IgG-2.

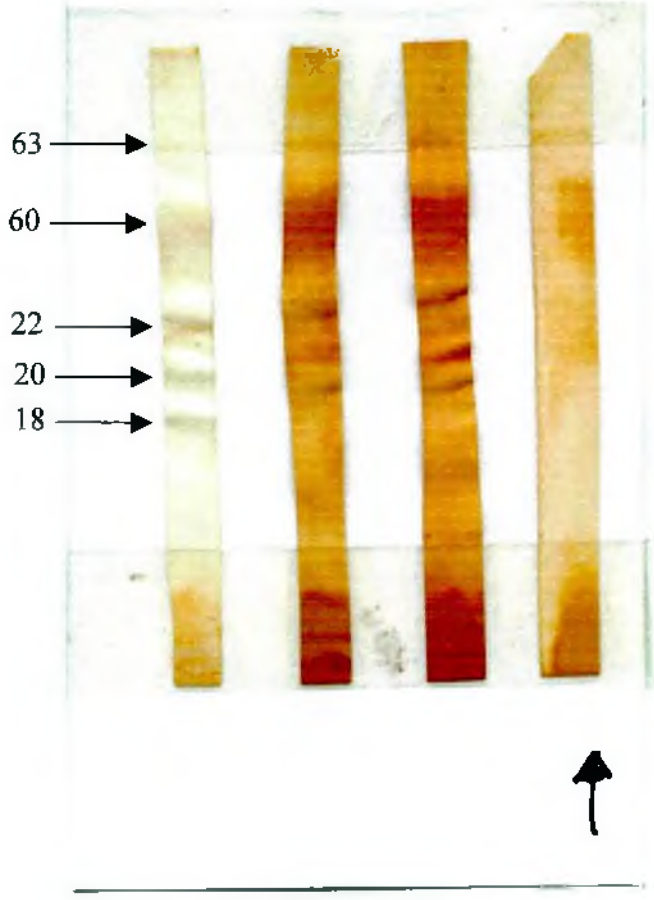

Figure –6: IgG1, IgG2, showed reactivity to 63, 60, 22, 20, 18KD, IgG3 showed reactivity 63, 60, 22, 21, 18KD and IgG4 fail to show any reactivity.

CHAPTER V

DISCUSSION

5. Discussion

Leishmania still remain, as endemic in some pockets of this country. It appears that Kala-azar is an emerging public health problem. A resurgence of KA was first noted in early 1970 when 59 parasitologically confirmed cases were reported from different parts of Bangladesh (Rahman *et al.,* 1983). To ensure proper control and to eradicate leishmaniasis regular prevalence survey is necessary. So a descriptive type of analytic study carried out in a Kala –azar endemic upazilla to know the present status of Kala –azar, to analyze *leishmania donovani* specific IgG subclass antibody, to find out the kinetics of IgG subclass profile, to do the analysis of leishmanial antigen, to assess the effectiveness of PCR and to determine the sero –epidemiology of Kala –azar in an endemic rural community.

Survey by bone-marrow examination is difficult. The better and convenient methods for detection of Leishmania are serological method but serological test cannot differentiate old and new cases and there are little study regarding IgG subclasses in different stages of the diseases.

A total of 449 households were visited and among them 1844 household members were selected for blood sample collection for sero-surveillance of Kala azar. The present study was carried out to investigate the sero-prevalence of kala-azar infection in an endemic rural community. Epidemiological data and blood samples were collected from 1844 people of the selected villages to determine the sero-prevalence of KA in an endemic rural community.

A recent study reported the incidence of KA as 0-9 cases/1000 population in the endemic areas of Bangladesh and 31 out 64 districts are already affected (Chowdhury et al, 1988; WHO report 1988). The data from CDC, DGHS shows an upward trend in the number of cases reported from 3965 in 1994 to 8846 in 1998 with slight decline during 1998-99 periods, then again rising to 7640 in the year 2000. The highest cases have been reported from Mymensingh followed by Pabna, Jamalpur, Sirajganj, and Natore. Our study revealed that the actual exposure rate is much greater then reported earlier. It is about 10% and the disease rate is 3.8% that indicate that the numbers of kala-azar cases are higher than reported. It is because that our study is the first systematic community based sero-survey of kala-azar. The reported cases of kala-azar were mainly from diseases surveillance report generated by the attending patients in the health complexes. The economically backward section of the rural community was most affected. The young and the adults were the most affected signifying the adverse effect of this disease on the workforce of our country. It was also found that there were tendency of clustering of the disease within the family. It is recommended that case detection by serological means is an effective method of detecting active cases and may play an important role in controlling the diseases. No public health program will be successful if effective laboratory tools and support are not available for ensuring correct detection of cases.

All the five union of the Fulbaria Upazilla were affected. The exposure rate and the active infection rate were determined serologically using a cut off value for exposure and active disease.

The study finding showed that out of 1844 patients 189 (10%) were seropositive cases by ELISA method. Among the sero- positive cases highest percentages (13.0%) were within the age group of 20-29 years followed by 11.2% in the age range of 30-39 years. Finding also revealed that female members are more (90.6%) then the male. But among the seropositive cases in the study 131 were active KA patients where 70 patients were from serosurvillence and 61 were from referred cases, where males (71.8%) were more than females. The study finding showed that out of 131 KA patient the highest number (29.8%) of the respondents was within the age groups of 10-19 years.

In a recent study conducted in IEDCR stated that there were two peak incidences of age groups, one with in the age range of 0-10 years and another with in the age range of 26-30 years (Begum *et al.*, 2002). In another study conducted by Talukder (2000) showed that the highest number (27.9%) of the patients of kala-azar were in the age group of $\leq$ 10 years (Talukder, 2000). Among the sero-positive cases, highest percentage (13.0%) were within the age group of 20-29 years flowed by 11.2% in the age range of 30-39 years, 11.1% were less than 10 years, 9.6% were in the age range of 50-59 years. The present

study may be regarded as consistent with the findings of the mentioned other studies.

Regarding the sex distribution of the sero-positive cases, it was found that among male members the sero-positive status was a bit higher (11.2%) then the female members (9.4%) but the difference was not statistically significant ($p>0.05$). This result was consistent with the study findings of IEDCR, which was 1.8:1 during 1996-1998 (Begum *et al.,* 2002). In another study by Talukder (2000) it was revealed that male-female ratio was 1.88:1 (Talukder, 2000). So, reviewing the present study and previous studies it may be commented that kala-azar is more common in male than female. However the present study does not show statistically significant association between sex of the respondents and sero-positive result ($p=0.1$).

In the present study among the 131 KA cases, most of their (80%) houses were mud-houses and followed by tin-houses (11.0%). The rest were thatched (6%). Another study revealed that 95.4% of Kala-azar patients live in Kacha houses (Hossain, 1998). One study showed that increased incidence of the disease was among those who lived in the houses with walls made of mud and cow-dung (Chowdhury *et al.,* 1993). From the above findings, it may be concluded that the dwelling in the mud houses is an important factor in occurrence of Kala-azar.

In the present study, sero-positive result for Kala-azar was found more among the students (33.8%), followed by farmers (22.3%). One of the reasons of the more affected number of students may be due to the poor socio-economic condition of the study area where majority of the students might have helped their parents in farming in addition to their study.

Kala-azar has an important relationship with occupation. The previous study carried by Talukder (2000) revealed that it was more common among farmers (25.9%), followed by students (20.8%). However in this study among 131 KA Kala-azar is more prevalent among students 44(33.8%) followed by farmers 29 (22.3%). One of the reasons of the differences may be that due to poor socioeconomic condition of the study area students might help their parents in farming in addition to their study.

LD bodies were demonstrated in 131 active cases of kala-azar. This high positivity rate by microscopy could be due to careful case selection. We have included only those cases, which had most florid clinical features of kala-azar. Though we have detected LD bodies in all cases, others have reported the detection rate being 61.1% previously (Alam *et al.,* 1996).We could not detect LD bodies in buffy coat by microscopy.

It was interesting to note that though LD bodies were present in all the 131 cases, the culture positivity rate was 74%. Culture was negative in 28 cases. The isolation rate of parasite by culture could be further increased if we could

culture the marrow specimens immediate after collection. Since some samples were collected in Gazipur General Hospital some time had elapsed to transport the culture tubes to the laboratory in Dhaka. However other workers from Bangladesh reported the isolation rate around 35% (Alam *et al.*, 1996).

All the 131 KA cases were positive for anti-leishmanial antibody as determined by ICT and ELISA methods. ICT was found to be very effective and easy to perform at the field level. But the cost of the test is high which would restrict its widespread use. The cost per test is around US$ 2.5.

In this study ELISA was tried to find the positive cases and plates were coated with K39 antigen for the detection of IgG. Detection of IgG and IgG subclasses were done. A declining trend of all the subclasses was noticed. The antibody level of all subclasses was determined after 15 days, 3 months, 6 months and 9 months. The declining kinetics of the subclasses were observed after treatment and the level of antibody do not touch the base line even after 9 month but declining nature of the antibody from the graph gives us the impression that antibody declines after treatment with the passage of time. So, in case have re infection and relapse cases we can get an idea about the patient. We can also understand from the graph that there is a great difference of OD between the control group and patient. In this study control group from endemic and non-endemic area were also considered with their value of standard deviation to

determine the mean OD. The Kinetics of OD was also analyzed by logistic regression.

In this study, IgG subclass assay shows that IgG1 & IgG3 antibody is not sensitive than IgG2, IgG4. IgG and subclasses decline in the serum of the patient after treatment with the passage of time. There is no significant decline of kinetics of IgG1 & IgG3 in comparison to IgG2 & IgG4 after treatment. This finding is a remarkable and new one. In determining the cut off value we considered the sera of *Leishmania* negative individuals from the endemic area. Since observing morphologies under microscope cannot differentiate *Leishmania* species, several methods have been developed for improvement of the parasite identification including genotypic and phenotypic approaches. Monoclonal antibody based ELISA has been reported to be an alternative method to identify the parasites at species level (Mimori *et al.*, 1989; Furuya *et al.*, 1998). These methods, however, need culture of the parasite, which is complex and laborious, and they are usually impeded by the bacterial and/or fungal contamination and slow growth of the Leishmania parasite in the culture media. Moreover, and it is a time consuming procedure. In the present study, all the cases were taken from Gazipur and Mymensingh district. It appears that Kala-azar is an emerging public health problem. The majority of our patients were children and young adult population signifying that the young population was the most affected or susceptible groups.

So PCR was tried and the primer set used in this study was able to amplify leishmania kDNA from buffy coat, bone marrow, and promastigote from culture and from sand fly. A series of recent studies have focused on the use of PCR amplification of kinetoplast DNA and nuclear DNA from different clinical samples (Samsuzzaman *et al.,* 2001). It has been reported that PCR was the most sensitive and specific tool among the methods available and it can detect very low quantity of DNA in a sample. We could detect kDNA in the buffy coat of peripheral blood by PCR in our parasitologically positive VL cases. The PCR became negative after treatment in the treated cases tested. The cases that were tested negative by PCR were also clinically cured. We have found that PCR of buffy coat from peripheral blood is a very effective, sensitive and non-invasive tool for diagnosis and monitoring the therapeutic effects of VL cases.

One of the problems in sero-diagnosis of kala-azar is that the serological tests remain positive for long period even after treatment. So, it becomes difficult to determine the effect of treatment by measuring the antibody. But ELISA can be used as a diagnostic tool for determining the antibody label, which can give us the direction whether it is a new or old infection, by detection of antibody label.

The conventional methods of diagnosing VL by detecting antibodies have some disadvantages. Firstly, detectable antibodies may not be found in early stages of the disease. Secondly, antibodies persist inside the body several years after

cure of VL, and thirdly, anti-*Leishmania* antibodies are found among residents of endemic areas who had no history of leishmaniasis. The second alternative may be antigen detection and the best method is to identify the parasite under microscope or in culture. But the sensitivity of these methods was low. So determination of *Leishmania* DNA may be the best alternative method of diagnosis of the cases as it is highly sensitive and specific. A series of recent studies have focused on the use of PCR amplificaiton of kinetoplast DNA and nuclear DNA from different clinical samples (Shamsuzzamans, *et al.,* 2001). It has been reported that PCR was the most sensitive and specific tool among the methods available and it can detect very low quantity of DNA in a sample.

PCR tried in identifying presence of parasite DNA inside the sandfly and it was found that *P. argentipies* contain the parasite DNA and gave the band in the same 354 bp as that of positive control. Mukherjee S et al observed similar finding, 1997 where they have detected *Leishmania donovani* DNA in sand flies caught in Indian Kala-azar patient's dwellings.

Now a days investigators are interested in DNA typing of leishmania species using nuclear kinetoplast DNA probes. In the present study the leishmania strains isolated from VL patients were identified as L.donovani where we have amplified a part of ITS of DNA from most of the Old World leishmania. WHO references strains using set of primers. The primers set used in this study was able to amplify that the DNA of most of the samples of buffy coat, bone marrow, promastigote from culture and even promastigote from sandfly. All

the amplified PCR products from different specimen showed band in the same region of 354 bp.

The findings correspond to the previous reports regarding identification of leishmania from Bangladesh (Shamsuzzaman *et al.*, 2001) and the PCR product showed the similar sequencing as that of Shamsuzzaman *et al.*, (2000). Other researcher also reported leishmania strain from Bangladeshi patient, which was characterized by iso-enzyme analysis (Shamsuzzaman *et al.*, 2000, El-Masum *et al.*, 1995). But the finding of Adhya *et al.*, (1995) showed that 28% of the patient were positive by PCR which are negative by direct Microscopy and 18% are positive in negative samples by all the methods. Nuzum *et al.*, (1995) showed the application of PCR on Kala-azar patient both at pre and post treatment stage and the finding was in similarity with our finding. Poonam *et al.*, 2001 showed that PCR could diagnose 95.3% cases with 95% sensitivity.

In conclusion the primer set used in this study was sufficient to amplify the DNA of most of the leishmania species from Bone marrow as well as blood (Buffy Coat), which is a non-invasive method and can be utilized as a tool for molecular epidemiological survey.

In the present study immunoblot reactive in the serum recognized certain immune bands around the 60 to 63 kDa and 28-kDa regions. The IgG-3 antibodies show broad reactivity to leishmania antigen predominantly in the region between 60, 63, 21, 22,18 and 34 kDa while the IgG-4 isotype filed to

show any significant reactivity to leishmanial antigen. In reactive phase, IgG-1 showed reactivity particularly at 60 , 63 kDa and 20 , 22 and 18 kDa and same with the IgG2.

In the study, specific antibody responses to L. donovani polypeptide in the immunoglobulin classes IgG1, IgG2, IgG3, & IgG4 were examined. We have examined in immunoblots the specificity of serum antibodies during L. donovani infection by using promastigote polypeptides separated electrophoretically by SDS-PAGE. There was also variability of the intensity of staining. (Dos santos *et al,* 1987; mary *et al*, 1992). This variability of recognition has been correlated with the individual genetic variations that exist within a population.

Leishmania parasites are known to express a 63 kD surface glycoprotein gp63 (Bouvier *et al,* 1985, colomes-gould *et al,* 1985) which is recognized by sera from patients with different forms of leishmaniasis including VL (Bogdan *et al,* 1990; Dos Santos *et al,* 1987; Heath *et al,* 1987; reed *et al,* 1987). This glycoprotein migrates on SDS-PAGE between 60 and 65 kD depending on the species of Leishmania (Wilson *et al,* 1989; Gardiner *et al,* 1984). The gp63 has been described being important in cell-cell (host-parasite) interaction (Russell *et al.* 1986; Wilson *et al,* 1988), cell infectivity (Kweider *et al,* 1989; Wilson *et al,* 1989) specific diagnosis (Heath *et al,* 1987) and immunoprotection (Yang *et*

al, 1990). The 56-64 kD band (s) in our immunoblot experiment is likely to arise as a result of recognition of this major surface glycoprotein in different stages of deglycosylation (Fong *et al,* 1982).

Attempts to identify *L. donovani* polypeptides recognized by visceral leishmaniasis sera in immunoblot experiments showed considerable variability in the pattern of antigen recognition by infected sera from different geographic region (Dos Santos *et al,* 1987; Reed *et al,* 1987; Bogdan *et al,* 1990; Rolland-Burger *et al,* 1991; Mary *et al,* 1992, Marty *et al,* 1995; Cardenosa *et al,* 1995). In our study we found that the most leishmania specific polypeptides were between the molecular mass ranges of 18 to 63 kD, while others have found bands within lower molecular mass range to be more specific (Mary *et al,* 1992, Cardenosa *et al,* 1995.) Dos Santos *et al,* (1987) found bands of 119 kD and 123 kD to be more specific. Strict comparisons between our result and other results reported in literature are rather difficult because of the variability of techniques, the use of different strain and the difference in the genetic make-up of the population suffering from VL. Although cross-reactive antigens are evident among the different *leishmania* species (Wilson *et al,* 1989; Colomer-Gould *et al,* 1985; Lepay *et al,* 1983), antigenic differences have also been documented (Lemesre *et al,* 1985; Gardiner *et al,* 1984). It has been observed in animal model that there is marked heterogeneity in antibody responses to specific antigens between different inbred strains of animal, which suggests the existence of genetic control (kee *et al,* 1986). Responses to some antigens were

found to be major histocompatibility complex (MHC) haplotype-restricted. While for other antigens, response may depend on genes outside the MHC (Kennedy *et al,* 1986).

The infected population involved may give rise to differences in parasite polypeptide recognition from different geographic region. Differences in preparation of promastigote extracts, membrane bound or surface antigens and soluble or cytoplasmic antigen, may also give different antigen recognition pattern with the same serum sample of VL patient (Tebourski *et al,* 1994).

A total of 131 patients were treated with Urea Stibamin and 129 (98.5%) were cured completely. The rest 2(1.5%) patients relapsed later on. But when they were given another cycle for 30 days they were cured completely.

The steady rise in Sb (V) unresponsiveness of KA patients in India is due to infection with resistant parasites, generated as a result of irregular and often incomplete treatment of the patients.

Urea Stibamin is the oldest antileishmanial compound and it's pentavalent compounds were remained the sole treatment modality but resistance even to it's higher doses (Singh S *et al* 2004) were also reported.

The magnitude of kala-azar problem is much more than the presently assumed. ELISA with well-calculated cut off value is an important tool for sero-epidemiology and determination of magnitude of the problem. PCR is an effective non-invasive tool for diagnostic and prognostic purpose. The study for the first time showed the presence of parasites in sand flies (the vector of parasite) of the endemic area. It is recommended that large-scale sero-survey may be undertaken to assess the extent of the diseases in the country.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion:

It was a descriptive type of analytic study carried out in a Kala –azar endemic upazilla to know the present status of Kala –azar, to analyze *leishmania donovani* specific IgG subclass antibody, to find out the kinetics of IgG subclass profile, to do the analysis of leishmanial antigen, to assess the effectiveness of PCR and to determine the sero –epidemiology of Kala –azar in an endemic rural community.

- In these study IgG and its subclass status for early and acute diagnosis is helpful to identify the prognosis and differentiate the recent and old infection. Quantitative detection of Ab is a good technique for diagnosis of KA. Declinig antibody titre after treatment indicates the effectiveness of the treatment.
- ICT is one of the sensitive and specific test as compare to ELISA , PCR, Direct microscopy and culture.
- Western blot analysis with IgG subclass specific reagent revealed variable pattern of reactivity and the reaction to IgG1 and IgG3 showed certain common band around 60 –63 KD regions with descernidy.
- PCR of Buffy coat is an effective noninvasive diagnostic tool and can be used to assess the success of treatment. The sensitivity of the PCR is 100% when parasite positive sample were tested. PCR can also be used for detection of *L. donovani* DNA from sand flies (*P. argentipes*). This is the first demonstration of *L.donovani* parasite in sand fly vector in Bangladesh.

- The study showed that the disease in more among male and occurs in the low socioeconomic groups. The most of the dwelling houses of the study area are mud houses and regarding occupation the diseases were found more among students.

6.2 Recommendations

The following recommendations are made in the light of the study for the prevention and control of kala –azar in Bangladesh.

- For rapid and accurate diagnosis of kala–azar, sensitive and specific serological field tests ICT should be made available at upazila and union level.
- Kinetics of IgG before and after treatment can be taken in consideration for the diagnosis and prognosis of Kala –azar.
- In future PCR can be used as an important tool for the diagnosis of Kala – azar from the blood samples. Further study should be encouraged in this field.
- Communities should be involved in sandfly control through the primary health care approach, cracks and crevices in the floor and walls of mud houses should be repaired, cattle shade should be away from their living room and rubbish should be removed from around the houses to reduce the number of sandfly breeding sites.

- Field health personnel as well as community leaders and volunteers should be trained, using primary health care approach, to recognize clinical kala – azar cases.
- As untreated or partially treated kala –azar patients act as a source of infection, follow up surveillance system for kala –azar patients should be developed.
- Vector control measures should be taken in endemic area because clustering characteristics of the disease.
- Regular surveillance, Seminar, workshop, training programme on kala – azar should be arranged in collaboration with neighboring countries, WHO and other organizations for elimination of kala-azar.
- More facilities regarding operational and experimental research should be made.

CHAPTER VII

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CHAPTER VIII

APPENDIX

6. Appendix

Appendix I: Reagents and Chemicals

Reagents Preparation

1. **Resolving Buffer**

a) 1.5 m Tris with 0.4 % SDS

Tris –Base	36.34g
SDS	0.80g
Distilled water	200 ml

b) 1.5 M Tris with 0.4 % SDS

Tris –HCL	47.30g
SDS	0.80g
Distilled water	200 ml

* Tris –Base	80 ml
Tris -HCL	20 ml

Adjust pH to 9.3

2. Stacking Buffer:

Tris-Base	6.05g
SDS	0.40g
Distilled water	40 ml
Adjust pH to 6.8	
Add water to 100 ml	

3. Running Buffer

Tris -Base	3.0 g
Glycine	14.4 g
SDS	1.0 g
Distilled water	1000 ml
Adjust pH to 8.3	

4. Sample Buffer

Tris –Base	0.76 g
Glycerol	10 ml
SDS	1.0 g

Adjust pH to 6.8 with HCl. Add water to 50 ml. Put 10 % mercaptoethanol when it's ready to use. Store at 4 °C.

5. Staining Solution:

Distilled water	325 ml
Isoprophanol	125 ml
Coommasie	1.0 g

6. Fixer Destaining Solution:

Acetic acid	300 ml
Isoprophanol alcohol	300 ml
Distilled water	2400 ml

7. Transfer Buffer:

Tris -Base	3.03 g
Glycine	14.4 g
Distilled water	1000 ml

Dissolve in ½ of actual ml of distilled water. Add 200 ml methanol (for IL) or 600 ml for 3 L. Make up to actual amount. 'Prechill' the buffer before use.

8. Phosphate Buffer saline

$Na_2HPO_4.2H_2O$	5.32 g
$NaH_2PO_4.2H_2O$	4.68 g
NaCl	4.383 g
Trimerosol	0.100 g
Distilled water	1000 ml

Adjust pH to 7.4

Autoclave

Store at 4 ° C

9. 10nM Tris HCl

Tris HCl	0.778 g
Distilled water	250 ml

Adjust pH to 7.4. Make up to 500 ml.

10. 1.0M of NaOH

Dissolve 40.0 g of NaOH pellets in distilled water and dilute to 1 Litre

Caution: Heat evolved

11. Acidified Glycine 0.2%

Glycine	0.3g
Distilled water	10 ml

Adjust pH to 2.2 with 1N HCl. Make up to 20 ml

12. Phosphate buffer saline (PBS) pH 7.2, 0.15 M

Sodium Chloride	8.00 g
Pottasium chloride	0.02 g
Na_2HPO_4 (0.008M)	1.15 g
Pottasium di –hydrogen phosphate	0.20 g

Dissolve in 1000 ml of distilled water

Store at 4 °C

Appendix II: Reagent for ELISA

Eagle's Minimum Essential Medium (EMEM) With Earle's Balanced Salt Solution (EBSS) (Hudeson & Hay, 1980)

Eagle's medium Power (Sigma, USA)	0.96 gm
Foetal Calf Serum* (Difco, USA)	20.0 ml
Penicilin	20,000 units
Streptomycin	1 mg
Deionised Distilled Water	100.0 ml

The solid ingredients were dissolved in distilled water and filtered using membrane filter (0.22μm, Sartorius) The meduum was then aseptically dispensed in sterile screw-capped test tubes, 10ml in each tube and stored at 4^0 C until use.

*Fetal calf serum was reconstituted by adding deionised water to dehydrated material according to the manufacturer's instruction.

Appendix III: Determination of Protein by Bradford's Method

REAGENTS:

1. Bradford's reagent (Bio-Rad)
2. Phosphate buffered saline (PBS)
3. Deinised water

Bradford's reagent was diluted 1:5 in deionised water prior to use.

PROCEDURE:

The reagents were mixed according to the chart below

REAGENTS	BLANK	SAMPLE
PBS	800µL	200µL
BRADFORD'S REAGENT (DILUTED)	200µL	200µL
SAMPLE	------	10µL
TOTAL VOLUME	**1000µL**	**1000µL**

Optical density (OD) was measured in a spectrophotometer using a 595 nm filter. The protein concentration was calculated by using the formula given below:

$Y = mx+c$

Where, Y=OD of test sample

m=0.024, x=micogram of protein in 10 micro litre of sample

c=0.121

Appendix IV: SDS-PAGE

Preparation of Buffers and Reagents used in SDS -PAGE

1. **30% Acrylamide Solution**

Acrylamide	60.0gms
Bis-acrylamide	1.64gms
Deionised water	200ml

The solid ingredients were dissolved in deionised water. The solution was then filtered through Whatman # 1 filter paper and stored at 4^0 C in a dark glass bottle.

2. **Lower (Resolving) Gel Buffer 1.5 Tris HCI, 0.4% SDS**

Tris (hydroxymethyl aminomethane), Bio-Rad	18.17 gms
Sodium Dodecyl Sulphate (SDS), Bio-Rad	0.4gm

The solid ingradients were dissolved in 75 ml of deionised water. The pH was adjusted to 8.8 with hydrochloric acid. The volume of the solution was then made upto 100ml. The buffer was the stored in a dark glass bottle at 4^0 C.

3. **Upper (Stacking) Gel Buffer 0.5M Tris HCI, 0.4% SDS**

Tris, Bio-Rad	6.06 gms
SDS	0.4 gms

The solid ingredients were dissolved in 75 ml of deionised water. The pH was adjusted to 6.8 with hydrochloric acid. The volume was made up to 100ml. The solution was then stored in a dark glass bottle at 4^0 C.

4. 10% Ammonium Persulphate Solution

Ammonium persulphate (APS), Bio-Rad	200mg
Deionised water	2ml

APS was dissolved in deionised water. 100µl aliquots were made and stored at-20^{0}C.

5. Sample Buffer 625mM Tris, 10% glycerol, 5% mercaptoethanol, 2.3% SDS, 0.001% bromophenol blue.

Tris	0.757 gm
Glycerol	10.0 gm
2-Mercaptoethanol	5.0 ml
SDS	2.3 gms
0.1% Bromophenol blue* (Sigma)	1 ml

The ingredients were dissolved in 75 ml of deionised water. The pH was adjusted to 6.8 with hydrochloric acid. The volume was made upto 100 ml. Small aliquots were made and stored at 4^0 C.

*** 0.1% Bromophenol Blue**

Bromophenol blue (Sigma)	10 mg
Deionised water	10 ml

The dye was dissolved in water to give a solution of 0.1% Bromophenol blue.

6. Running Buffer 250Mm Tris, 1992mM glycine, 0.1% SDS

For 10 times concentration

Tris	30.0 gms
Glycine	144.0 gms
SDS	10 gms

The ingredients were dissolved in 750 ml of deionised water. The volume was made upto 1000 ml. The pH should be 8.2 – 8.5 and should not be adjusted. If the pH was off by 0.2, the buffer was discarded. The buffer was stored at 4^0 C.

For preparing 2.5l single strength running buffer

Stock running buffer (x10)	250 ml
Deionised water	2250 ml were mixed together

Appendix V: Electro blotting

1. Transfer Buffer 250Mm Tris, 192 mM glycine, 20% methanol

For 10x concentration stock solution

Tris	30.03 gms
Glycine	144.0 gms

To prepare 3l of single strength transfer buffer

Stock solution	300 ml
Methanol	600 ml
Deionised water	2100 ml

Were mixed together. The buffer was chilled before use.

Appendix VI: Immunostaining

1. **1% BSA IN PBS**

Bovine serum albumin (BSA), Sigma	1 gm
PBS	100 ml

BSA was dissolved in PBS. Sterile container was used.

2. **0.1% BSA in PBS**

1% BSA in PBS	10 ml
PBS	90 ml

3. **0.05% Tween 20 in PBS**

Tween 20	250µl
PBS	500ml

4. **0.1% BSA IN PBS-Tween 20**

1% BSA in PBS	2 ml
PBS-Tween 20	18 ml

5. **Veronal Acetate Buffer**

0.3M veronal acetate buffer stock solution

5,5- diethyl- barbituric acid sodium salt	12.37 gms.
Deinised water	200 ml

The salt was dissolved in 150 ml of deionised water. The pH was adjusted to 9.6 with 1M acetic acid. The volume was made up to 200ml.

To make 0.15M veronal acetate buffer, equal volumes of stock solution and deionised water were mixed together.

6. Substrate

BCIP	2.5 mg
Nitroblue tetrazolium	5.0 mg
0.15M veronal acetate buffer	45 ml
1.0M $MgCl_2$	200µl

BCIP was dissolved in 100µl.

NBT was dissolved in 2.5 ml of deionised water using a vortex mixer. Then 2.5 ml of 0.3M veronal acetate buffer added to it.

Finally, to prepare the substrate solution, 100µl. of BCIP soution, 5.0 ml of NBT solution, 200µl. 1.0M $MgCl_2$ and 45.0 ml 0.15M veronal acetate buffer was added together. The substrate was prepared freshly just before use.

7. Sample Diluents:

1 Vial phosphate buffered saline with Triton X –100 as surfactant,

0.05% Kathon CG as preservative and bovine serum as stabilizer.

8. Conjugate Diluents:

1 vial phosphate saline –casein buffer with 0.05% Kathon CG as preservative

Substrate Concentrate (101X):

1 vial TMB (Tetramethyl Benzidine) in DMSO (Dimethyl Sulfoxide)

Appendix VII: PCR Reagents

1. TE Buffer

Tris –Base	0.12 gm
EDTA	0.029 gm
Distilled water	100 ml

Adjust pH 8.2

2. AW1 Buffer

Guanidiana hydrochaloride

Distilled water

Ethanol

3. AL Buffer

Guanidiana hydrochaloride

Distilled water

4. Proteinase K

Enzyme

5. AE Buffer

Used for elution

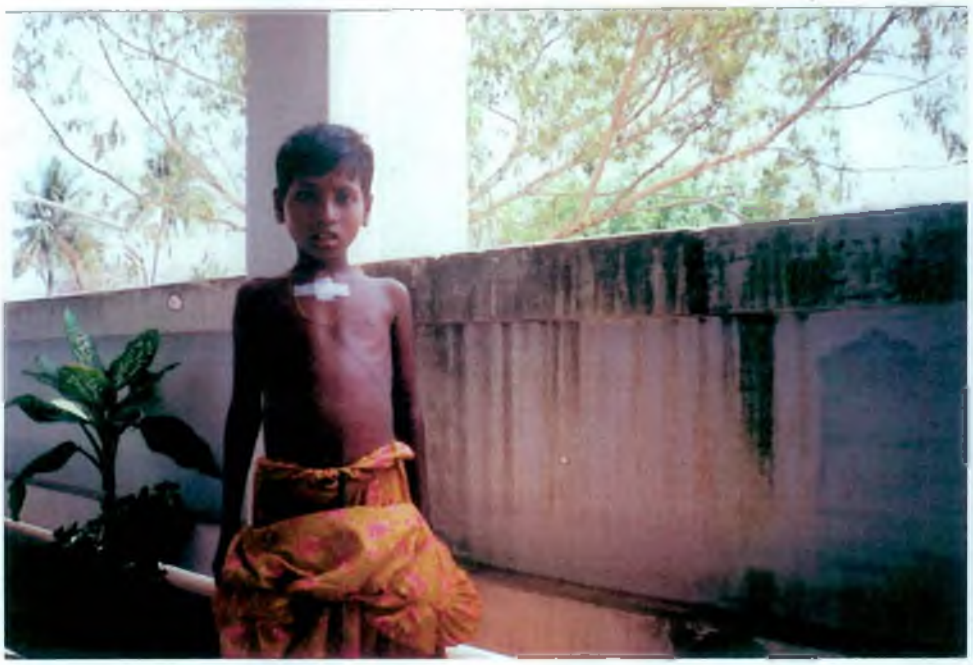

Figure 9. Kala - azar patient after bone marrow aspiration

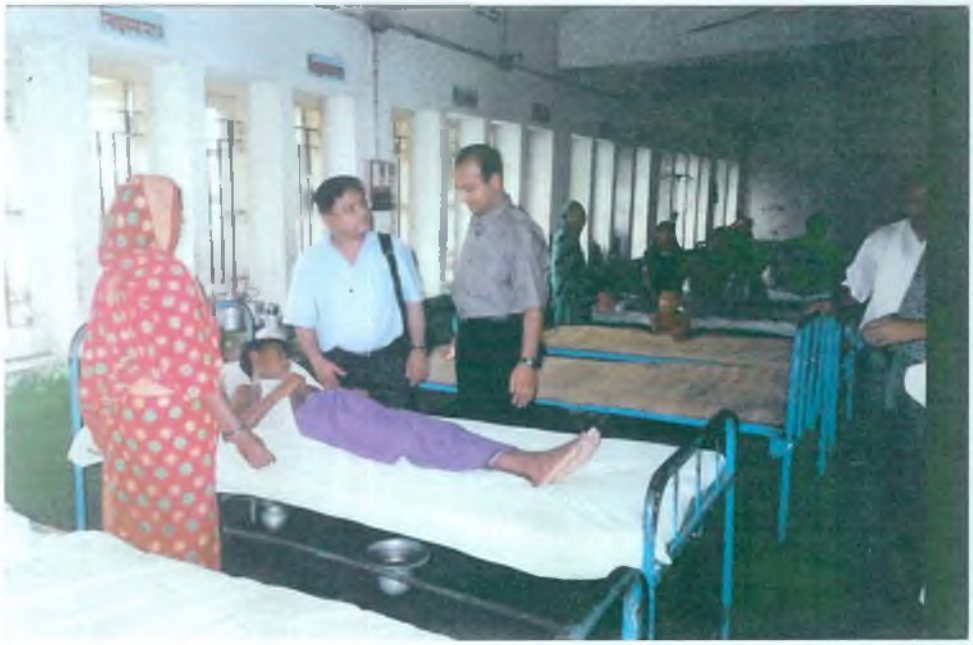

Figure 10. Kala - azar patient receiving treatment in Fulbaria

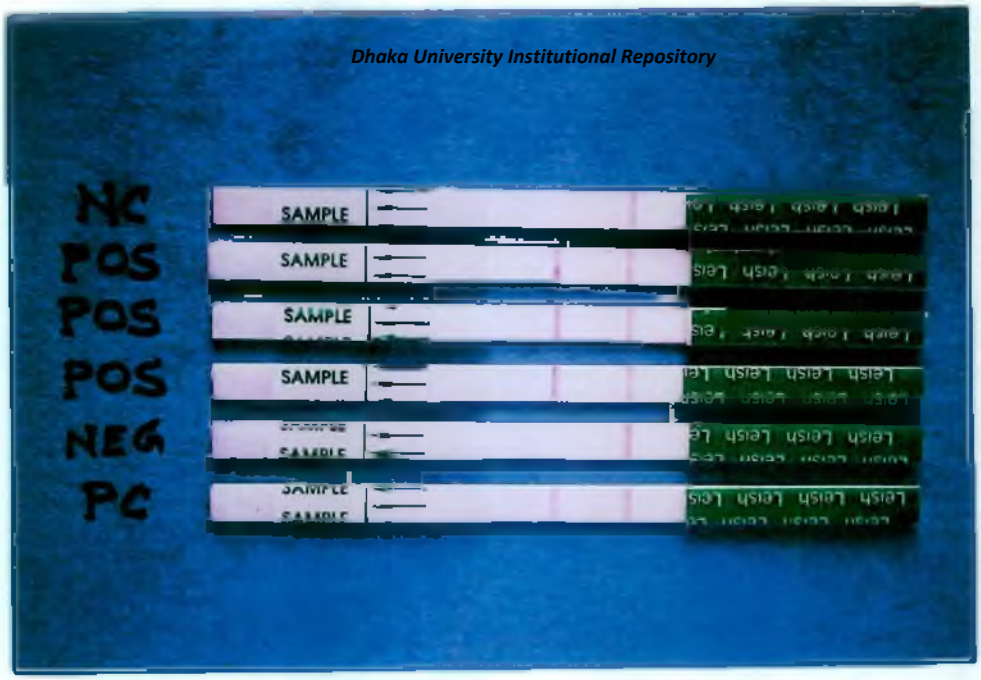

Figure-11 ICT field test of Kala –azar patient showing positive and negative bands

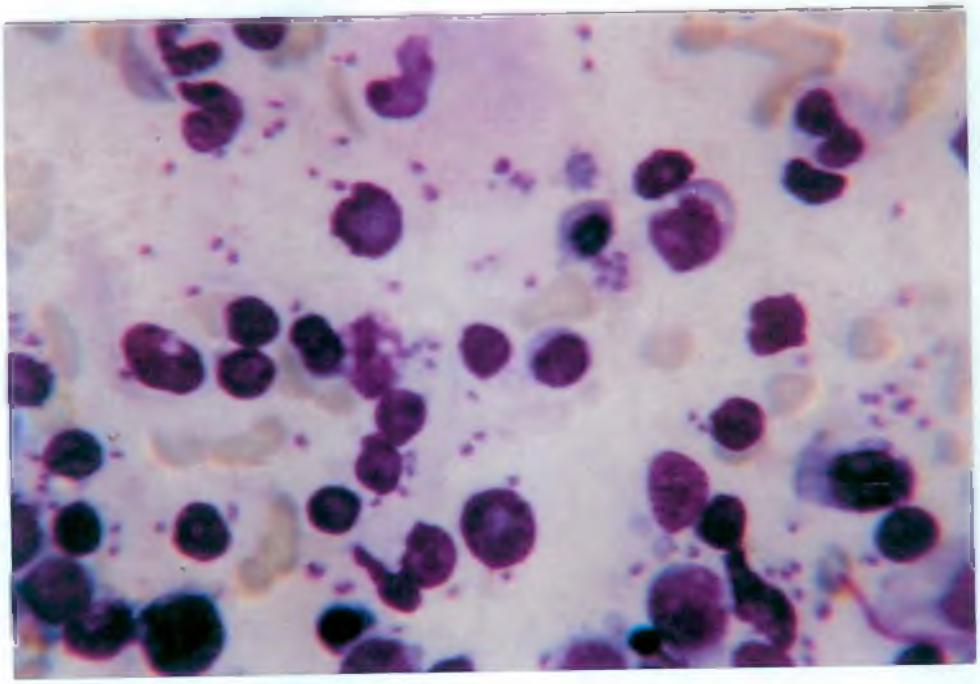

Figure 12 Amastigote form of LD bodies in bone marrow aspiration slide

Figure-13 NNN media showing the culture of *L. donovani*

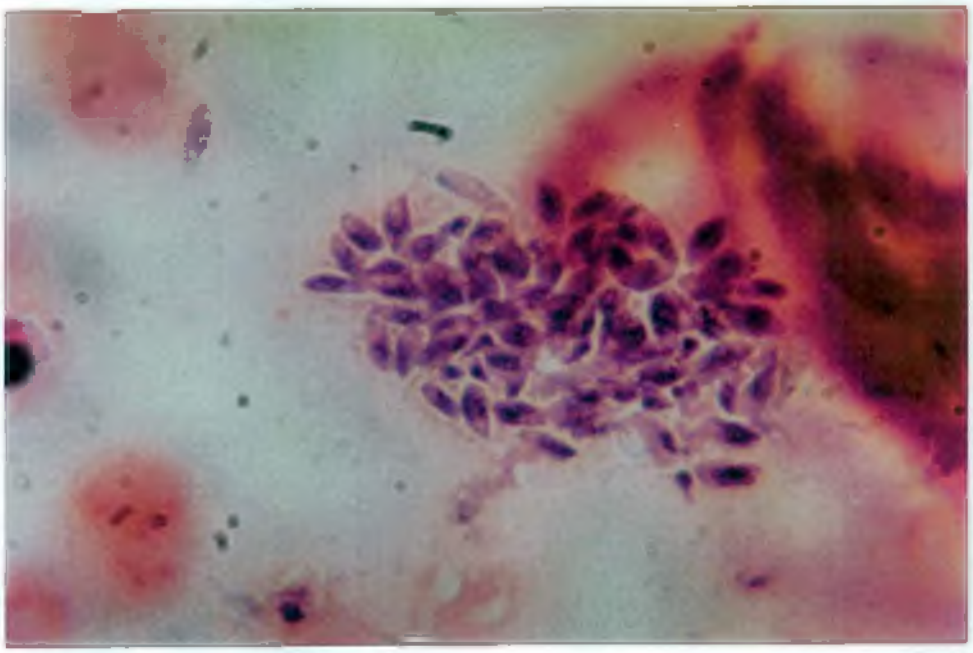

Figure 14 Promastigote form of *L. donovani*

Figure-15

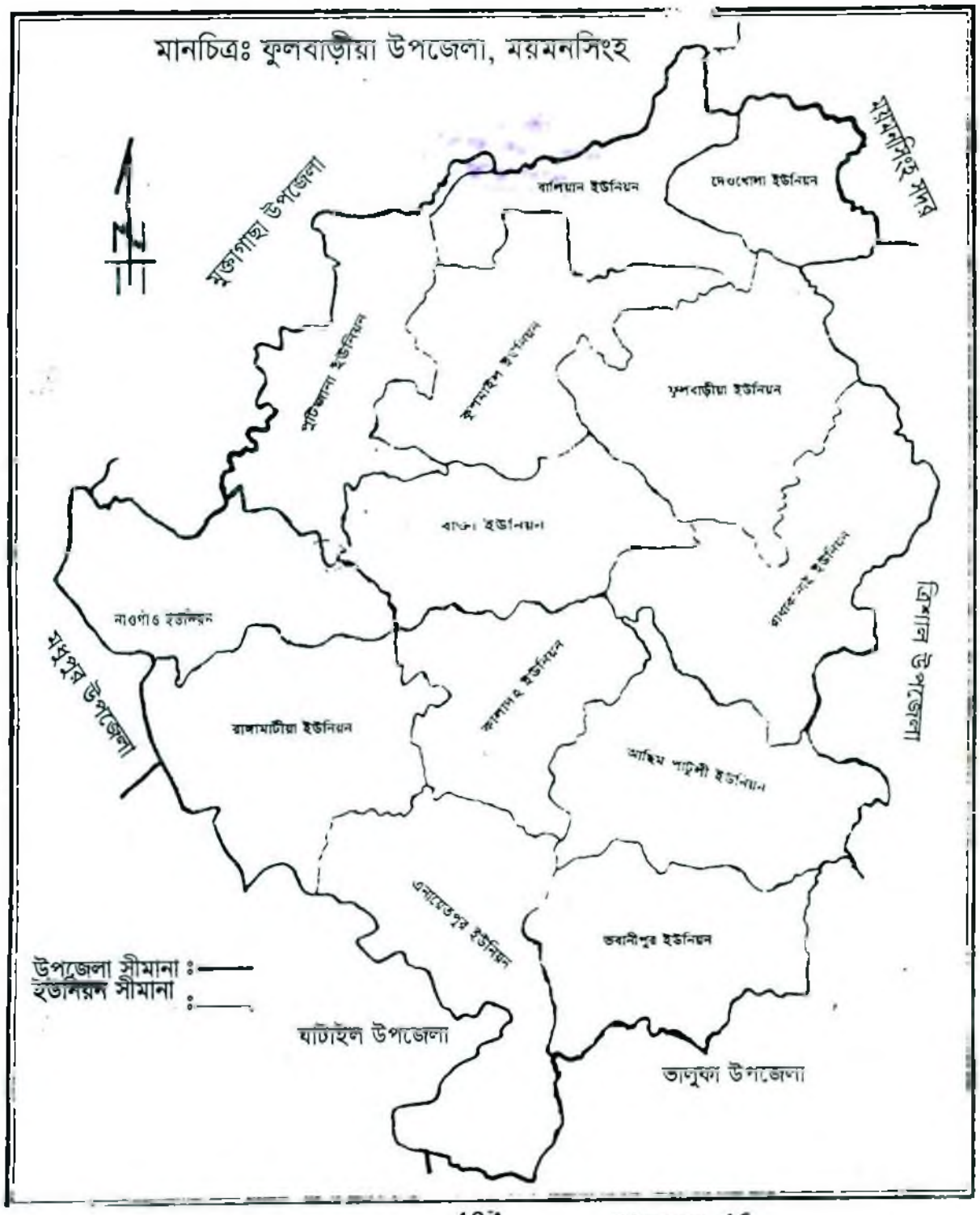

 Figure-16